SATHYABAMA
INSTITUTE OF SCIENCE AND TECHNOLOGY
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SCHOOL OF BIO AND CHEMICAL ENGINEERING

DEPARTMENT OF BIOTECHNOLOGY

UNIT – I – Basic Industrial Biotechnology – SBB2202

1. Fermentation

Fermentation is the process involving the biochemical activity of organisms, during their growth, devel-opment, reproduction, even senescence and death. Fermentation technology is the use of organisms to produce food, pharmaceuticals and alcoholic beverages on a large scale industrial basis.

The basic principle involved in the industrial fermentation technology is that organisms are grown under suitable conditions, by providing raw materials meeting all the necessary requirements such as carbon, nitrogen, salts, trace elements and vitamins.

The end products formed as a result of their metab-olism during their life span are released into the media, which are extracted for use by human being and that have a high commercial value. The major products of fermentation technology produced econom-ically on a large-scale industrial basis are wine, beer, cider, vinegar, ethanol, cheese, hormones, antibiotics, complete proteins, enzymes and other useful products.

Fermentation Methodology:

Fermentation process is carried out in a container called the fermentor or bioreactor. The design and nature of the fermentor varies depending upon the type of fermentation carried out. Invariably all the fermentors have facilities to measure some of the fermentation parameters like temperature, pressure, pH, elapsed fermentation time, liquid level, mass etc.

The different types of fermentors are:

1. External recycle airlift fermentor—for producing bacterial biomass, with methanol as substrate.
2. Internal recycle airlift fermentor—for producing yeast with oil as substrate.
3. Tubular tower fermentor—Used for making beer, wine, vinegar etc.
4. Nathan fermentor—used in brewing industry.
5. Stirred fermentor—used for making antibiotics.

Types of Fermentation Processes:

1. Batch Fermentation: A batch fermentation is a closed culture system, because initial and limited amount of sterilized nutrient medium is introduced into the fermenter. The medium is inoculated with a suitable microorganism and incubated for a definite period for fermentation to proceed under optimal physiological conditions. Oxygen in the form of air, an antifoam agent and acid or base, to control the pH, are being added during the course of fermentation process.

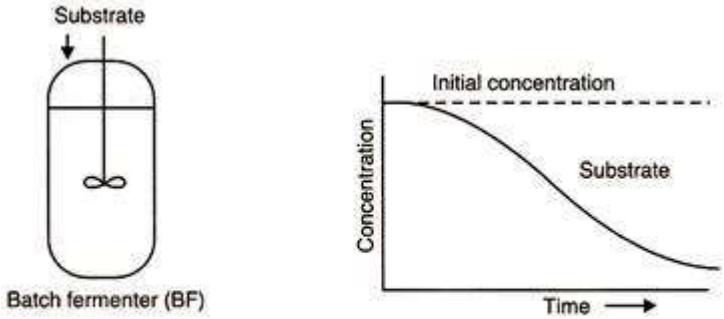
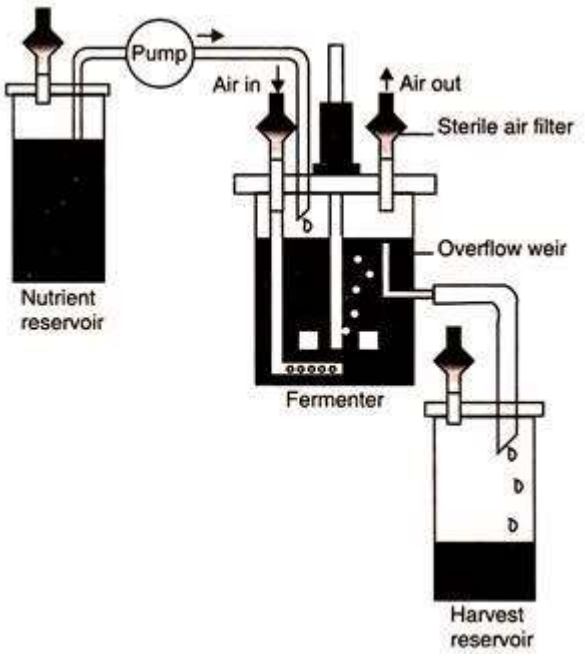


Fig. 2.11: A typical batch fermenter

During the course of incubation, the cells of the microorganism undergo multiplication and pass through different phases of growth and metabolism due to which there will be change in the composition of culture medium, the biomass and metabolites. The fermentation is run for a definite period or until the nutrients are exhausted. The culture broth is harvested and the product is separated. Batch fermentation may be used to produce biomass, primary metabolites and secondary metabolites under cultural conditions supporting the fastest growth rate and maximum growth would be used for biomass production. The exponential phase of growth should be prolonged to get optimum yield of primary metabolite, while it should be reduced to get optimum yield of secondary metabolites. The used medium along with cells of microorganism and the product is drawn out from the fermenter. When the desired product is formed in optimum quantities, the product is separated from the microorganism and purified later on.

2. Continuous Fermentation: It is a closed system of fermentation, run for indefinite period. In this method, fresh nutrient medium is added continuously or intermittently to the fermenter and equivalent amount of used medium with microorganisms is withdrawn continuously or intermittently for the recovery of cells or fermentation products



As a result, volume of the medium and concentration of nutrients at optimum level are being maintained. This has been operated in an automatic manner. The continuous fermenter has its maximum use that take long time to reach high productivity, reduces down time and lowers the operating costs. In continuous mode, starting medium and inoculum are added to the fermenter. After the culture is grown the fermenter is fed with nutrients and broth is withdrawn at the same rate maintaining a constant volume of broth in the fermenter. In continuous mode with cell cycle, the cell mass is returned to the fermenter using micro filtrations with bacteria or screens with fungal mycelium.

A continuous fermentation is generally carried out in the following ways:

1. Single Stage Fermentation: In this process, a single fermenter is inoculated and the nutrient medium and culture are kept in continuous operation by balancing the input and output of nutrient medium and harvested culture, respectively.

2. Recycle Fermentation: In this method, a portion of the medium is withdrawn and added to the culture vessel. Thus, the culture is recycled to the fermentation vessel. This method is generally adopted in the hydrocarbon fermentation process. The recycling of cells provides a higher population of cells in the fermenter which results in greater productivity of the desired product.

3. Multiple Stage Fermentation: In this process, two or more fermenters are employed simultaneously and the fermentation is operated in a sequence. Different phases of fermentation process like growth phase and synthetic phase are carried out in different fermenters. Generally, growth phase is allowed in the first fermenter, synthetic phase in the second and subsequent fermenters.

The process of continuous fermentation is monitored either by microbial growth activity or by product formation and these methods are called:

1. Turbidostat Method: In this method the total cell content is kept constant by measuring the culture turbidity at a regular interval of fermentation process. By turbidity measurement it is possible to the fermenter to regulate both the nutrient feed rate and the culture withdrawal rate.

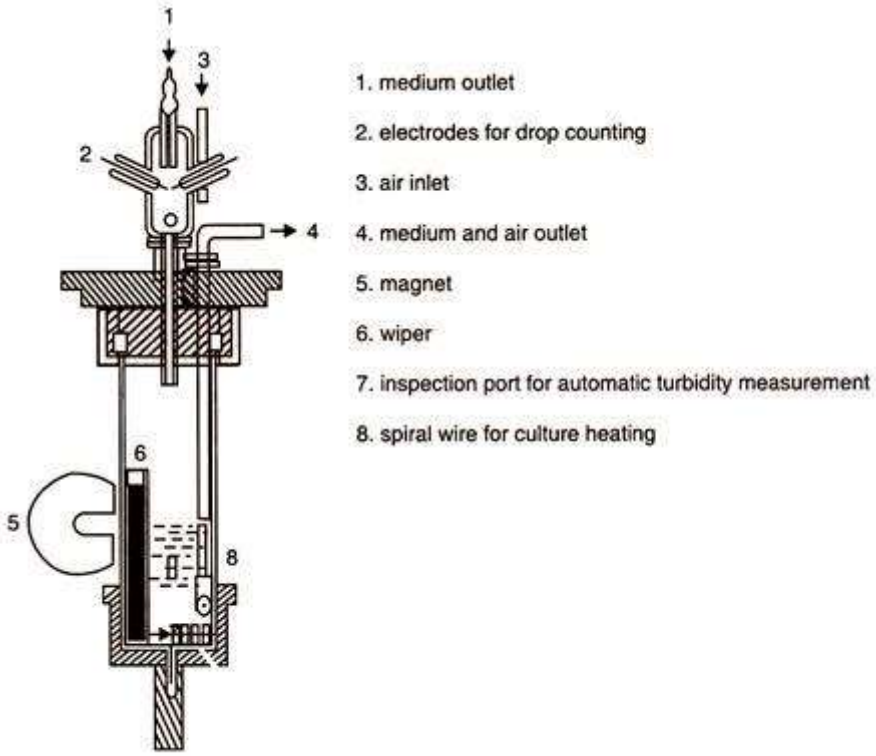


Fig. 2.13: Turbidostat

Fermentation, in which this method is employed, must be carried out at a low maximum cell population which leads to the usage of less amount of substrate and wastage of greater amount of substrate as unused and residual medium, which is removed from the fermenter along with the harvested culture

2. Chemostat Method: In this method nutrient feed rate and harvest culture withdrawal rate are maintained at constant value. This is achieved by controlling the growth rate of the microorganism by adjusting the concentration of any one of the chemicals of the medium, like carbon source, nitrogen source, salts, O_2 etc. which acts as a growth limiting factor. Apart from the above chemicals, sometimes the concentration of the toxic product generated in the fermentation process, the pH values and even temperature also act as growth limiting factors. This method is employed more often than turbidostat method because of fewer mechanical problems and presence of less amount of unused medium in the harvested culture

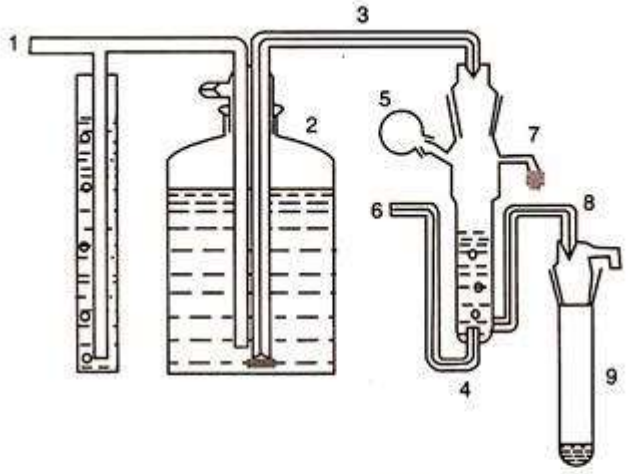


Fig. 2.14: Chemostat

1. Air inlet	2. Mariotte's bottle	3. Capillary for medium inlet
4. Culture vessel	5. Inoculation port	6. Air inlet
7. Air outlet	8. Overflow capillary	9. Sampling tube

3. Fed Batch Fermentation: It is a modification to the batch fermentation. In this process substrate is added periodically in instalments as the fermentation progresses, due to which the substratum is always at an optimal concentration. This is essential as some secondary metabolites are subjected to catabolite repression by high concentration of either glucose, or other carbohydrate or nitrogen compounds present in the medium. For this reason, the critical elements of the nutrient medium are added in low amount in the beginning of the fermentation and these substrates continue to be added in small doses during the production phase. This method is generally employed for the production of substances such as penicillin. Yoshida (1973) introduced this term for the first time for feeding the substrates to the medium as the nutrients are exhausted, so as to maintain the nutrients at an optimum level.

II. Basic concepts of Upstream and Downstream processing in Bioprocess

The current topics of debate on the Basic concepts of Upstream and Downstream processing in Bioprocess:

1. Up-Stream Process: This process comprises of obtaining a desired microorganism, and its improvement so as to enhance the productivity and yield. It also includes the maintenance of strain purity, preparation of inocula and further efforts to improve the economic efficiency of the process. There are two distinct processes involved in USP but both the processes run simultaneously. The medium is prepared for the culture (inoculum) preparation as well as for the production of desired product. The vessel of the fermenter is filled with production media and inoculated with suitable microbial culture for the industrial product formations as shown in Fig. 20.4.

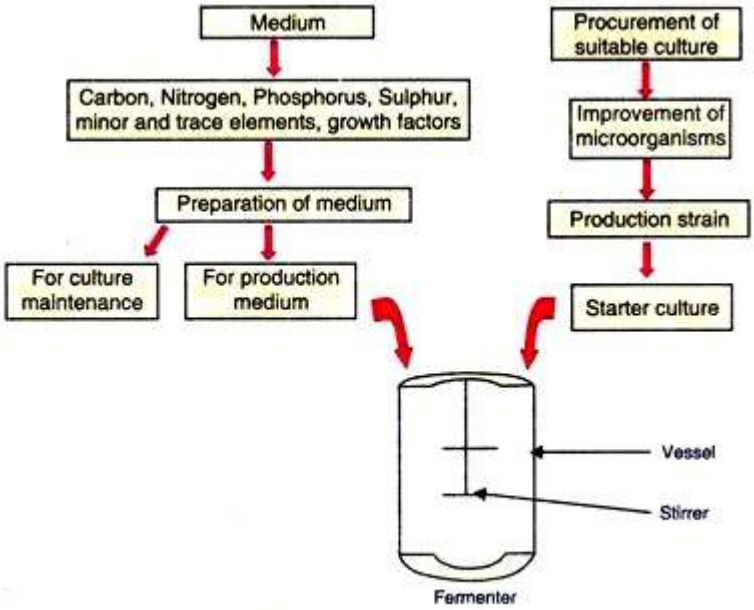


Fig. 20.4: Steps of up-stream process (USP).

2. Down-Stream Process: This process includes the selection of suitable fermentation media, optimization of important fermentation condition to give rise maximum yield. The main objective of this process is to get reproducibility of result as far as possible besides safe recovery of the target product. Further processing is carried out depending upon whether the product is intracellular or present inside the cell or membrane bound or release outside the cell or extracellular in nature. This leads to recovery of the product in pure form. The presence of undesirable products (by products), impurities in media, antifoam agents, etc. affect the DSP

steps. Therefore, each step is required thorough check up in both USP as well as in DSP. For example, a cheap carbon and energy source may increase DSP cost. At commercial level use of existing equipment's, ancillaries, etc. are more advantageous than that of creating new facilities because the later step requires more investment which may lead to escalation of cost of the product and processes. The DSP consists of series of distinct unity or processes which are connected to each other for getting the final product as shown in Fig 20.5.

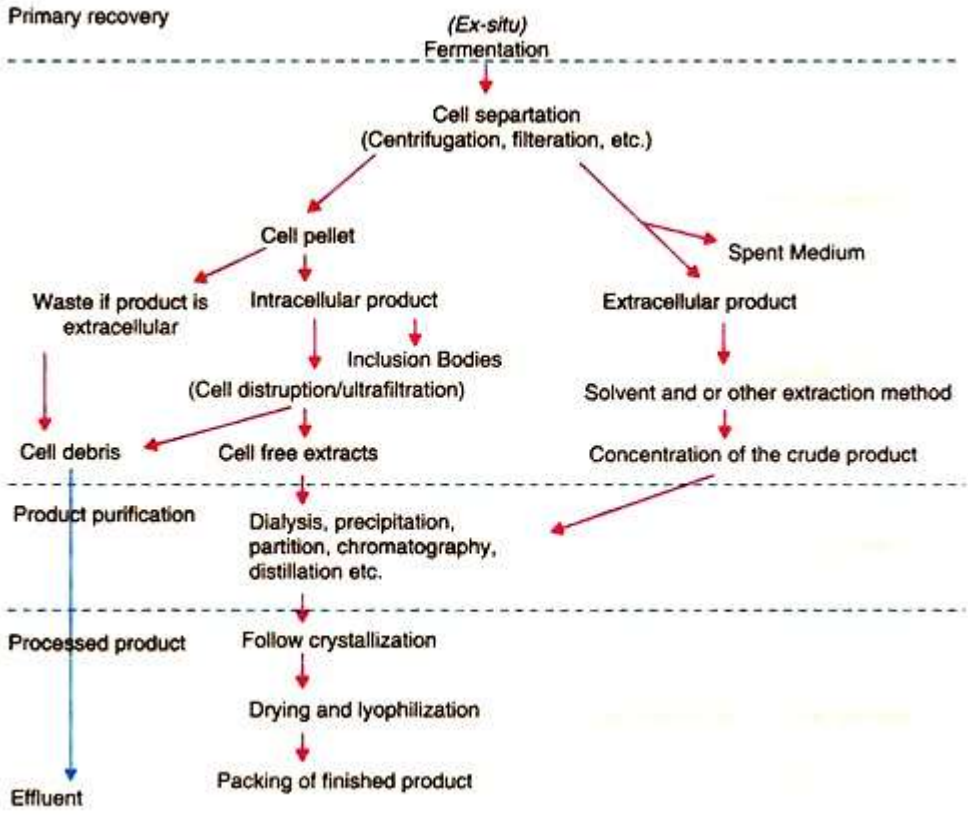


Fig. 20.5: Steps of down-stream process (DSP).

For the precautionary measures it is necessary to keep this unit process minimum. This will not only save the cost of the plant but will also ensure not to loose the product during individual steps (Fig. 20.5). Now-a-days, most preferred DSP is to link with the fermentation. This increases productivity and reduce time and cost. There are two types of major processes for product formation. The product formation is coupled with growth i.e. primary metabolites and another where product is formed after growth as in case of antibiotics. In case of primary metabolites, higher productivity is achieved by using integrated system of fermentation with DSP. This kind of system maintains a high cell density through cell retention of recycling. If product is inhibitory, various methods have been employed to partition fermenter as in case of formation of glucose by breakdown of cellulose. This allows the rapid in-situ removal of product by extraction, adsorption or stripping. The process can be ex-situ where the product is

removed outside the fermenter and the processed medium is returned to the fermentation. Such processes have been successfully used for removing alcohols, solvents, proteins, etc. as shown in Fig. 20.5. Following are the major steps for the product recovery.

1. Cell Harvesting: Since, microbial cells are in liquid medium or broth, solid-liquid separation method is influenced by the size and morphology of the microorganisms (single cells, aggregates or mycelia). Further, the process also depends upon the location of product whether it is intracellular or extracellular it has been secreted into the medium. Besides, other factors such as specific gravity, viscosity, and rheology of the medium also influence cell harvesting method.

2. Broth Conditioning Technique: This technique allows separation of cells from large volume of liquid medium. In this case, cells are allowed to form floccules or precipitate by using chemical, physical and biological treatments. Coagulating materials such as simple electrolytes, acids, bases, salts, multivalent ions etc. are added to form small floes from dispersed colloids or suspended materials. Certain floes precipitation methods are also used at the end of many traditional beer and wine fermentation processes for the precipitation of yeast cells.

3. Sedimentation: This process is widely used for yeast separation during alcohol production and in waste-water treatment. The rate of particle sedimentation is a function of both size and density. If, larger the size, greater its density, and the faster rate of sedimentation. Therefore, for quick separation, the difference in density between the particle and the medium must be large with low viscosity.

4. Centrifugation: This process allows small particles or cells to separate from liquid. It is also suitable for some liquid-liquid separations. Similar to sedimentation, its effectiveness also depends upon cell size, density difference between the cells and the medium, and its viscosity. Higher speed of centrifugation requires for the separation of smaller microorganisms, such as bacteria. In case of yeast cells present in beer, relatively, low centrifugation effectively recovers residual yeast cells.

5. Filtration: There are certain filters such as clothes, glass wool or cellulose that retain the solids and pass on the liquid. The solids accumulate above the filter. Such technique is

useful in case of fungal mycelia separation. There are two types of filters used in industrial process: plate and frame filters or filter press, and rotatory vacuum filter, which help in harvesting the fungal mycelia during antibiotic manufacture, bakers yeast production and in dewatering of sewage sludge.

6. Disruption of Microbial Cells: When the product is intracellular, it is necessary to disrupt the cells/cell membrane so as to release the product. Cell disruption can be achieved by mechanical and non-mechanical methods. Some of the methods used are ultra-sonication and application of lysozyme. The 'French-press' is often used in laboratories, while Manton and Gaulin homogenizer (high pressure homogenizer) is employed for pilot scale cell disruption. These are used for disruption of bacterial, yeast cells and fungal mycelia. The non-mechanical cell disruption is carried out by cell permeabilization. This can be accompanied by autolysis, osmotic shock, ruptures by ice crystal or heat shock treatment. Some organic solvents such as acetone, butanol, chloroform and methanol have been used to release enzymes from microorganisms. Simple treatment with sodium dodecyl sulfate (SDS) or Triton X-100 is also found effective. Lysozyme is useful for Gram-positive organisms but addition of EDTA improves the effectiveness of lysozyme of Gram-negative bacteria. Some antibiotics namely penicillin and cycloserine may also be used to lyse the actively growing bacterial cells. Chitosan is effective for yeast cells.

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1. Primary Metabolites

Primary metabolism, also referred to as trophophase, is characterized by balanced growth of microorganisms. It occurs when all the nutrients needed by the organisms are provided in the medium. Primary metabolism is essential for the very existence and reproduction of cells. In the trophophase, the cells possess optimal concentrations of almost all the macromolecules (proteins, DNA, RNA etc.).

It is during the period of trophophase, an exponential growth of microorganisms occurs. Several metabolic products, collectively referred to as primary metabolites, are produced in trophophase (i.e., during the period of growth).

The primary metabolites are divided into two groups:

1. Primary essential metabolites: These are the compounds produced in adequate quantizes to sustain cell growth e.g. vitamins, amino acids, nucleosides. The native microorganisms usually do not overproduce essential primary metabolites, since it is a wasteful exercise. However, for industrial overproduction, the regulatory mechanisms are suitably manipulated.

2. Primary metabolic end products: These are the normal and traditional end products of fermentation process of primary metabolism. The end products may or may not have any significant function to perform in the microorganisms, although they have many other industrial applications e.g. ethanol, acetone, lactic acid. Carbon dioxide is a metabolic end product of Saccharomyces cerevisiae. This CO_2 is essential for leavening of dough in baking industry.

Limitations in growth:

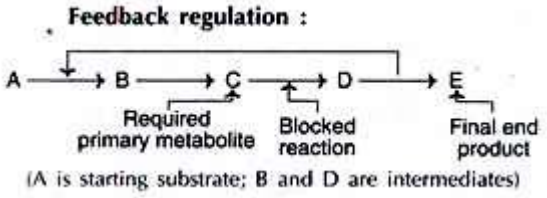
Due to insufficient/ limited supply of any nutrient (substrate or even O2), the growth rate of microorganisms slows down. However, the metabolism does not stop. It continues as long as the cell lives, but the formation of products differs.

Overproduction of primary metabolites:

Excessive production of primary metabolites is very important for their large scale use for a variety of purposes.

Overproduction of several metabolites has been successfully accomplished by eliminating the feedback inhibition as briefly described below:

1. By using auxotrophic mutants with a block in one of the steps in the biosynthetic pathway concerned with the formation of primary metabolite (this should be an intermediate and not the final end product). In this manner, the end product (E) formation is blocked, hence no feedback inhibition. But overproduction of the required metabolite (C) occurs as illustrated below.



(A is starting substrate; B and D are intermediates)

   In the above example, an un-branched pathway is shown. This type of manipulation for overproduction of metabolites can be done for branched metabolic pathways also.

2. Mutant microorganisms with antimetabolite resistance which exhibit a defective metabolic regulation can also overproduce primary metabolites.

Production of commercially important primary metabolites:

1. Gluconic Acid:

    1. Gluconic acid can be produced by several bacteria and fungi. Glucose, on a simple direct dehydrogenation, forms D-gluconolactone which is then converted to gluconic acid.

    2. Applications of Gluconic Acid:
        1. Gluconic acid is used in the manufacture of metals, stainless steel and leather, as it can remove the calcareous and rust deposits.
        2. It is used as an additive to foods and beverages.
        3. Gluconic acid has pharmaceutical applications — calcium and iron therapy.
        4. Sodium gluconate is used as a sequestering agent in many detergents.
        5. Gluconate is used for desizing polyester or polyamide fabrics.
        6. It is utilized in the manufacture of highly resistant (to frost and cracking) concrete.

    3. Microbial Production of Gluconic Acid:
        1. Gluconic acid can be produced by a wide variety of prokaryotic and eukaryotic microorganisms.
        2. Bacterial species of the genera— Gluconobacter, Acetobacter, Pseudomonas, Vibrio.
        3. Fungal species of the genera—Aspergillus, Penicillium, Gliocladium.

    4. Principle of production:
        1. The enzymatic reactions for the formation of gluconic acid in Gluconobacter suboxidans (bacteria) and Aspergillus niger (fungus) are depicted in Fig. 24.6.

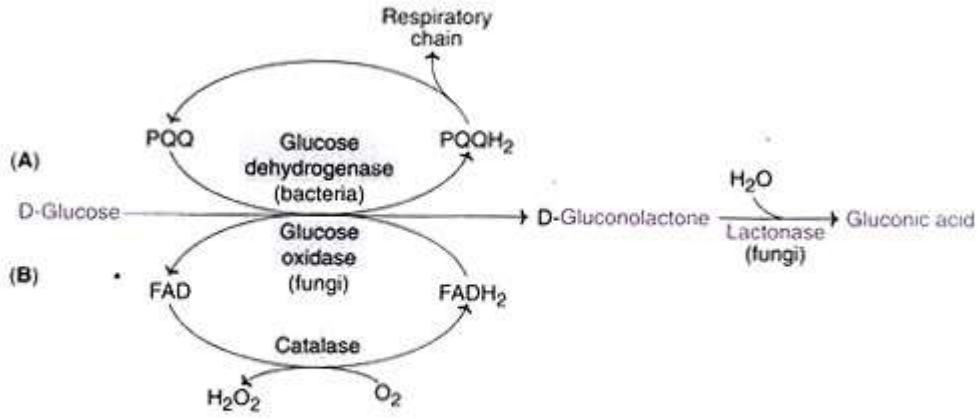


Fig. 24.6 : Biosynthesis of gluconic acid (A) in Gluconobacter suboxidans (B) in Aspergillus niger. (PQQ-Pyrroloquinoline quinone)

2. In bacteria, intracellular glucose is converted to extracellular gluconic acid. A membrane bound enzyme, glucose dehydrogenase utilizes pyrroloquinoline Quinone (PQQ) as coenzyme and converts glucose to 5-D-gluconolactone which undergoes hydrolysis (spontaneous or enzymatic) to form gluconic acid.

3. As regards fungal production, glucose is oxidized by the extracellular enzyme glucose oxidase to form 8-D-gluconolactone, which subsequently gets converted to gluconic acid by lactonase. Glucose oxidase is an inducible enzyme that can be induced by high concentrations of glucose, and at pH above 4. It is believed that H2O2 produced by glucose oxidase acts as an antagonist against other microorganisms (antimicrobial activity) in the surroundings.

2. Lactic Acid:

1. Lactic acid occurs in two isomeric forms i.e. L (+) and D (-) isomers, and as a racemic mixture (DL-lactic acid). The isolation of lactic acid from milk was done in 1798. It was the first organic acid produced by microorganisms in 1880. Today, lactic acid is competitively produced both by microbiological and chemical methods.

2. Applications of Lactic Acid:

1. There are different grades of lactic acid mainly based on the percentage of lactic acid. The grades and their applications are given in Table 24.2.

Table 24.2 Commercial grades of lactic acid along with their applications

Grade (% lactic acid)	*Application(s)*
Technical grade (20–50%)	Ester manufacture, textile industry
Food grade (>80%)	Food additive (sour flour and dough)
Pharmaceutical grade (>90%)	Intestinal treatment (metal ion lactates)

3. Microorganisms for Production of Lactic Acid:

    1. Hetero-fermentative bacteria—produce other byproducts, besides lactic acid, and therefore are not useful for industrial production of lactic acid. These bacteria are employed in food or feed preservation.

    2. Homo-fermentative bacteria—specialised for exclusive production of lactic acid and therefore are suitable for industrial purpose.

4. Production Process for Lactic Acid:

1. The fermentation medium contains 12-15% of glucose, nitrogen and phosphate containing salts and micronutrients. The process is carried out at pH 5.5-6.5 and temperature 45-50°C for about 75 hours. Generally, the strains operating at higher temperature (45-60°C) are preferred, since it reduces the need for medium sterilization.

2. As the lactic acid is produced, it has to be removed since it is toxic to the organisms. This can achieved either by a continuous culture technique or by removal of lactic acid by electro dialysis. Theoretically, every molecule of glucose forms two molecules of lactic acid. About 90% of theoretical yield is possible in fermentation industry. L(+) Lactic acid is predominantly produced. The outline of the steps involved in the recovery lactic acid is depicted in Fig. 24.8.

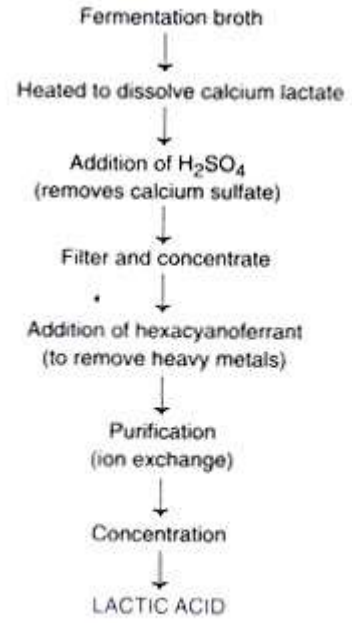


Fig. 24.8 : Flow chart for recovery of lactic acid from fermentation broth.

II. Secondary Metabolites

As the exponential growth of the microorganisms ceases (i.e. as the trophophase ends), they enter idiophase. Idiophase is characterized by secondary metabolism wherein the formation of certain metabolites, referred to as secondary metabolites (idiolites) occurs. These metabolites, although not required by the microorganisms, are produced in abundance. The secondary metabolites however, are industrially very important, and are the most exploited in biotechnology e.g., antibiotics, steroids, alkaloids, gibberellins, toxins.

Characteristics of secondary metabolites:

1. Secondary metabolites are specifically produced by selected few microorganisms.
2. They are not essential for the growth and reproduction of organisms from which they are produced.
3. Environmental factors influence the production of secondary metabolites.
4. Some microorganisms produce secondary metabolites as a group of compounds (usually structurally related) instead of a single one e.g. about 35 anthracyclines are produced by a single strain of Streptomyces.
5. The biosynthetic pathways for most secondary metabolites are not clearly established.
6. The regulation of the formation of secondary metabolites is more complex and differs from that of primary metabolites.

Functions of secondary metabolites:

Secondary metabolites are not essential for growth and multiplication of cells. Their occurrence and structures vary widely. Several hypotheses have been put forth to explain the role of secondary metabolites, two of them are given below.

1. The secondary metabolites may perform certain (unknown) functions that are beneficial for the cells to survive.
2. The secondary metabolites have absolutely no function. Their production alone is important for the cell, whatever may be the product (which is considered to be useless).

Overproduction of secondary metabolites:

As already stated, the production of secondary metabolites is more complex than primary metabolites. However, the regulatory manipulations employed for excess production of primary metabolites can also be used for the secondary metabolites as well.

Several genes are involved in the production of secondary metabolites. Thus, around 300 genes participate in the biosynthesis of chlortetracycline while 2000 genes are directly or indirectly involved in the production of neomycin. With such complex systems, the metabolic regulation is equally complex to achieve overproduction of secondary metabolites. Some regulatory mechanisms are briefly discussed hereunder.

Induction:

Addition of methionine induces certain enzymes and enhances the production of cephalosporin. Tryptophan regulates ergot alkaloid biosynthesis.

End product regulation:

Some of the secondary metabolites inhibit their own biosynthesis, a phenomenon referred to as end product regulation e.g. penicillin, streptomycin, puromycin, chloramphenicol. It is possible to isolate mutants that are less sensitive to end product inhibition, and in this manner the secondary metabolite production can be increased.

Catabolite regulation:

In this regulation process, a key enzyme involved in a catabolic pathway is inactivated, inhibited or repressed by adding a commonly used substrate. Catabolic repression can be achieved by using carbon or nitrogen sources. The mechanism of action of catabolite regulation is not very clearly understood.

The most commonly used carbon source is glucose. It is found to inhibit the production of several antibiotics e.g. penicillin, streptomycin, bacitracin, chloramphenicol, puromycin. The nitrogen sources such as ammonia also act as catabolite regulators (i.e. inhibitors) for the overproduction of certain antibiotics.

Phosphate regulation:

Inorganic phosphate (Pi) is required for the growth and multiplication of prokaryotes and eukaryotes. Increasing Pi concentration (up to 1 mM) is associated with an increased production of

secondary metabolites e.g. antibiotics (streptomycin, tetracycline), alkaloids, gibberellins. However, very high Pi concentration is inhibitory, the mechanism of action is not very clear.

Auto regulation:

In some microorganisms (particularly actinomycetes), there occurs a self regulation for the production of secondary metabolites. A compound designated as factor A which is analogous to a hormone is believed to be closely involved in auto regulation for the production streptomycin by Streptomyces griseus. More such factors from other organisms have also been identified.

Production of commercially important secondary metabolites:

01. An antibiotic is a type of antimicrobial substance active against bacteria. It is the most important type of antibacterial agent for fighting bacterial infections, and antibiotic medications are widely used in the treatment and prevention of such infections. They may either kill or inhibit the growth of bacteria. A limited number of antibiotics also possess antiprotozoal activity. Antibiotics are not effective against viruses such as the common cold or influenza; drugs which inhibit viruses are termed antiviral drugs or antivirals rather than antibiotics.

02. Sometimes, the term antibiotic—literally "opposing life", from the Greek roots ἀντι anti, "against" and βίος bios, "life"—is broadly used to refer to any substance used against microbes, but in the usual medical usage, antibiotics (such as penicillin) are those produced naturally (by one microorganism fighting another), whereas nonantibiotic antibacterials (such as sulfonamides and antiseptics) are fully synthetic.

03. However, both classes have the same goal of killing or preventing the growth of microorganisms, and both are included in antimicrobial chemotherapy. "Antibacterials" include antiseptic drugs, antibacterial soaps, and chemical disinfectants, whereas antibiotics are an

important class of antibacterials used more specifically in medicine and sometimes in livestock feed.

04. Antibiotics have been used since ancient times. Many civilizations used topical application of mouldy bread, with many references to its beneficial effects arising from ancient Egypt, Nubia, China, Serbia, Greece, and Rome. The first person to directly document the use of moulds to treat infections was John Parkinson (1567–1650). Antibiotics revolutionized medicine in the 20th century.

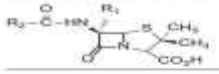
05. Penicillin:

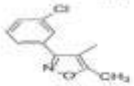
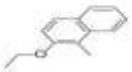
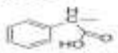
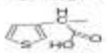
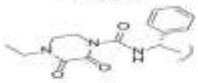
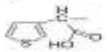
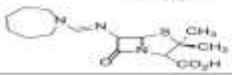
a. Penicillin G (benzylpenicillin) was the first β-lactam to be used clinically, most frequently to treat streptococcal infections for which it had high potency (Rammelkamp and Keefer 1943; Hirsh and Dowling 1946). Another naturally occurring penicillin, penicillin V (phenoxymethylpenicillin), in an oral formulation is still used therapeutically and prophylactically for mild to moderate infections caused by susceptible Streptococcus spp., including use in pediatric patients (Pottegard et al. 2015).

b. However, the selection of penicillin-resistant penicillinase-producing staphylococci in patients treated with penicillin G led to decreased use of this agent, and prompted the search for more penicillins with greater stability to the staphylococcal β-lactamases (Kirby 1944, 1945; Medeiros 1984).

c. A list of historically important and clinically useful penicillins is provided in Table 2. Among the penicillinase-stable penicillins of clinical significance are methicillin, oxacillin, cloxacillin, and nafcillin, with the latter suggested as the β-lactam of choice for skin infections, catheter infections, and bacteremia caused by methicillin-susceptible S. aureus (Bamberger and Boyd 2005). All were used primarily for staphylococcal infections until the emergence of methicillin-resistant S. aureus (MRSA) in 1979–1980 (Hemmer et al. 1979; Saroglou et al. 1980).

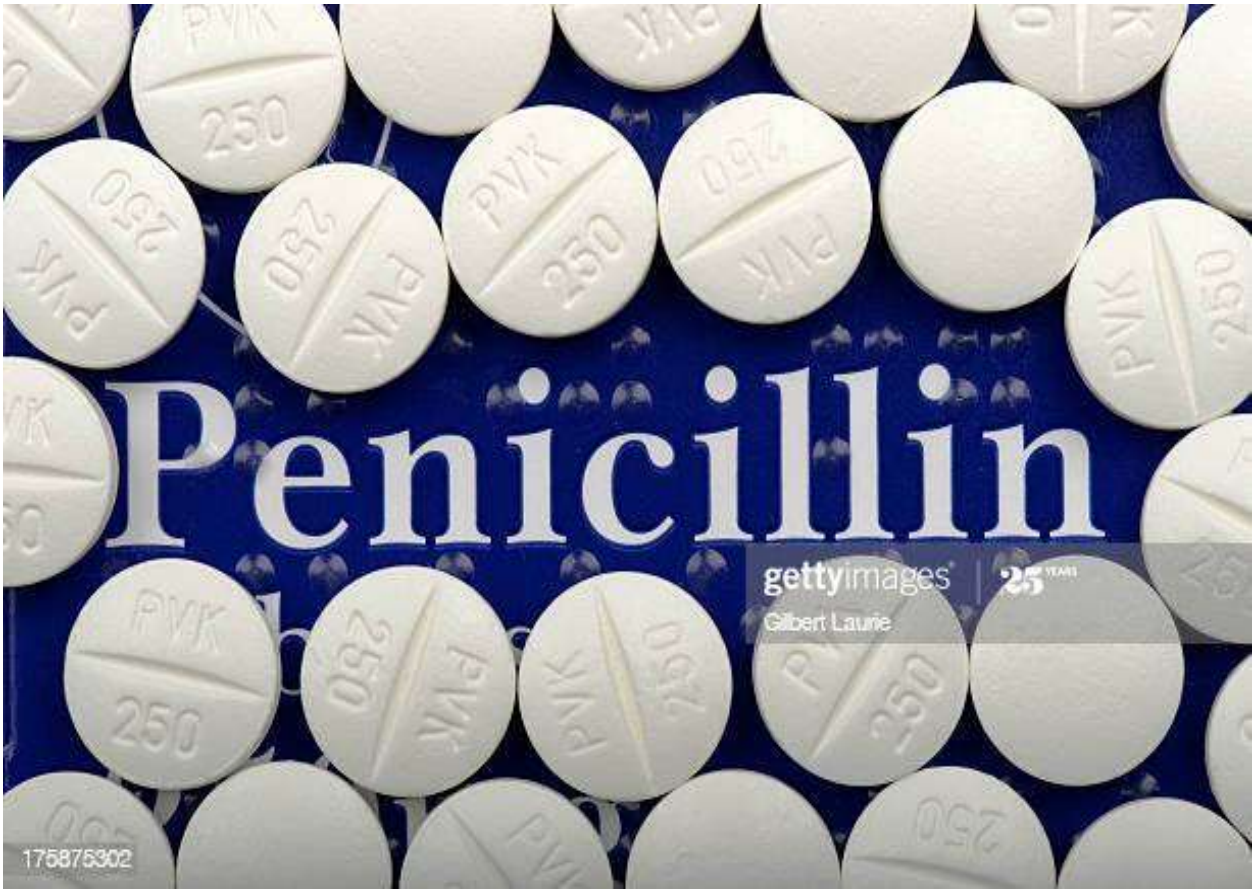
Table 2. Penicillins of current and historical utility



Name	R_1	R_2	Route of administration	Approval date[b,c]	Status
Benzylpenicillin (penicillin G)	—H		IM or IV	1946	Approved worldwide
Phenoxymethylpenicillin (penicillin V)	—H		Oral	1968	Approved worldwide
Methicillin	—H		IV	1960	No longer available; of historical interest
Oxacillin	—H		Oral, IV	1962	Widely available, but not in the United Kingdom
Cloxacillin	—H		Oral, IV	1974	Widely available, but not in the United Kingdom
Ampicillin	—H		Oral, IV	1963	Widely available
Nafcillin	—H		IV	1970	Limited availability
Amoxicillin	—H		Oral, IV	1972	Widely available
Carbenicillin	—H		Oral	1972	Discontinued
Ticarcillin	—H		IV	1976	Limited availability
Piperacillin	—H		IV	1981	Widely available, primarily in combination with tazobactam
Temocillin	—OCH_3		IV	1985 in Europe (Harvengt 1985)	Limited availability (Europe)
Mecillinam			IV	1978	Limited availability

IM, Intramuscular; IV, intravenous.
[b]FDA approval unless otherwise noted.
[c]Dates were updated from Medeiros (1997) (www.accessdata.fda.gov/scripts/cder/drugsatfda; www.drugs.com).

d. Penicillins with improved activity against Gram-negative pathogens included the orally bioavailable ampicillin and amoxicillin, both of which were introduced in the 1970s. These agents were initially used for the treatment of infections caused by Enterobacteriaceae and did not effectively inhibit the growth of Pseudomonas aeruginosa, which became more of a concern during the late 1970s.


Penicillin
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Gilbert Laurie
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SCHOOL OF BIO AND CHEMICAL ENGINEERING

DEPARTMENT OF BIOTECHNOLOGY

UNIT – III – Basic Industrial Biotechnology – SBB2202

1. Role of Microbes in degradation of Pollutants

Microorganisms are widely distributed on the biospher because of their metabolic ability is very impressive and they can easely grow in a wide range of environmental conditions. The nutritional versatility of microorganisms can also be exploited for biodegradation of pollutants. This kind of process is termed as bioremediation. It is continued through based on the ability of certain microorganisms to convert, modify and utilize toxic pollutants in order to obtaining energy and biomass production in the process [1]. Instead of simply collecting the pollutant and storing it, bioremediation is a microbiological well organized procedural activity which is applied to break down or transform contaminates to less toxic or non-toxic elemental and compound forms. Bioremediators are biological agents used for bioremediation in order to clean up contaminated sites. Bacteria, archaea and fungi are typical prime bioremediators [2]. The application of bioremediation as a biotechnological process involving microorganisms for solving and removing dangers of many pollutants through biodegradation from the environment. Bioremidation and biodegradation terms are more interchangable words. Microorganisms are act as a significant pollutant removal tools in soil, water, and sediments; mostly due to their advantage over other remediation procedural protocols. Microorganisms are restoring the original natural surroundings and preventing further pollution [3].

Factors affecting microbial bioremediation

Bioremediation is involved in degrading, removing, altering, immobilizing, or detoxifying various chemicals and physical wastes from the environment through the action of bacteria, fungi and plants. Microorganisms are involved through their enzymatic pathways act as biocatalysts and facilitate the progress of biochemical reactions that degrade the desired pollutant. Microorganisms are act against the pollutants only when they have access to a variety of materials compounds to help them generate energy and nutrients to build more cells. The efficiency of bioremediation depends on many factors; including, the chemical nature and concentration of pollutants, the physicochemical characteristics of the environment, and their availability to microorganisms [4]. The reason for rate of degradation is affected due to bacteria and pollutants do not contact each other. In addition to this, microbes and pollutants are not uniformly spread in the environment. The controlling and optimizing of bioremediation processes is a complex system due to many factors. These factors are included here: the existence of a microbial population capable of degrading the

pollutants, the availability of contaminants to the microbial population and environment factors (type of soil, temperature, pH, the presence of oxygen or other electron acceptors, and nutrients).

Biological factors

Abiotic factors are affect the degradation of organic compounds through competition between microorganisms for limited carbon sources, antagonistic interactions between microorganisms or the predation of microorganisms by protozoa and bacteriophages. The rate of contaminant degradation is often dependent on the concentration of the contaminant and the amount of "catalyst" present. In this context, the amount of "catalyst" represents the number of organisms able to metabolize the contaminant as well as the amount of enzymes(s) produced by each cell. The expression of specific enzymes by the cells can increase or decrease the rate of contaminant degradation. Furthermore, the extent to contaminant metabolism specific enzymes must be participated and their "affinity" for the contaminant and also the availability of the contaminant is largely needed. The major biological factors are included here: mutation, horizontal gene transfer, enzyme activity, interaction (competition, succession, and predation), its own growth until critical biomass is reached, population size and composition [5,6].

Environmental factors

The metabolic characteristics of the microorganisms and physicochemical properties of the targeted contaminants determine possible interaction during the process. The actual successful interaction between the two; however, depends on the environmental conditions of the site of the interaction. Microorganism growth and activity are affected by pH, temperature, moisture, soil structure, solubility in water, nutrients, site characteristics, redox potential and oxygen content, lack of trained human resources in this field and Physico-chemical bioavailability of pollutants (contaminant concentration, type, solubility, chemical structure and toxicity). This above listed factors are determine kinetics of degradation [5,7]. Biodegradation can occur under a wide-range of pH; however, a pH of 6.5 to 8.5 is generally optimal for biodegradation in most aquatic and terrestrial systems. Moisture influences the rate of contaminant metabolism because it influences the kind and amount of soluble materials that are available as well as the osmotic pressure and pH of terrestrial and aquatic systems [8].

Availability of nutrients

The addition of nutrients adjusts the essential nutrient balance for microbial growth and reproduction as well as having impact on the biodegradation rate and effectiveness. Nutrient balancing especially the supply of essential nutrients such as N and P can improve the biodegradation efficiency by optimizing the bacterial C: N: P ratio. To survive and continue their microbial activities microorganisms need a number of nutrients such as carbon, nitrogen, and phosphorous. In small concentrations the extent of hydrocarbon degradation also limit. The addition of an appropriate quantity of nutrients is a favourable strategy for increasing the metabolic activity of microorganisms and thus the biodegradation rate in cold environments [9,10]. Biodegradation in aquatic environment is limited by the availability of nutrients [11]. Similar to the nutritional needs of other organisms, oil-eating microbes also require nutrients for optimal growth and development. These nutrients are available in the natural environment but occur in low quantities [12].

Temperature

Among the physical factors temperature is the most important one to determining the survival of microorganisms and composition of the hydrocarbons [13]. In cold environments such as the Arctic, oil degradation via natural processes is very slow and puts the microbes under more pressure to clean up the spilled petroleum. The sub-zero temperature of water in this region causes the transport channels within the microbial cells to shut down or may even freeze the entire cytoplasm, thus, rendering most oleophilic microbes metabolically inactive [12,14]. Biological enzymes are participated in the degradation pathway have an optimum temperature and will not have the same metabolic turnover for every temperature. Moreover, the degradation process for specific compound need specific temperature. Temperature also speed up or slow down bioremediation process because highly influence microbial physiological properties. The rate of microbial activities increases with temperature, and reaches to its maximum level at an optimum temperature. It became decline suddenly with further increase or decrease in temperature and eventually stop after reaching a specific temperature.

Concentration of oxygen

Different organisms require oxygen others also do not require oxygen based on their requirement facilitate the biodegradation rate in a better way. Biological degradation is carried out in aerobic and anaerobic condition, because oxygen is a gaseous requirement for most living organisms. The presence of oxygen in most cases can enhance hydrocarbon metabolism [12].

Moisture content

Microorganisms require adequate water to accomplish their growth. The soil moisture content have adverse effect in biodegradation agents.

pH

pH of compound which is acidity, basicity and alkalinity nature of compound, it has its own impact on microbial metabolic activity and also increase and decrease removal process. The measurement of pH in soil could indicate the potential for microbial growth [15]. Higher or lower pH values showed inferior results; metabolic processes are highly susceptible to even slight changes in pH [16].

Site characterization and selection

Sufficient remedial investigation work must be performed prior to proposing a bioremediation remedy to adequately characterize the magnitude and extent of contamination. This work should at a minimum encompass the following factors: fully determine the horizontal and vertical extent of contamination, list the parameters and locations to be sample and the rationale for their choice, describe the methods to be used for sample acquisition and analysis to be performed.

Metal ions

Metals are important in small amount for bacteria and fungus, but in high quantity inhibit the metabolic activity of the cells. Metal compounds have direct and indirect impact on rate of degradation.

Toxic compounds

When in high concentrations of toxic nature of some contaminants, can create toxic effects to microorganisms and slow down decontamination. The degree and mechanisms of toxicity vary with specific toxicants, their concentration, and the exposed microorganisms. Some organic and inorganic compounds are toxic to targeted life forms [5].

Principle of 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. Microorganisms are suited to the task of contaminant destruction because they possess enzymes that allow them to use environmental contaminants as a food. The aim of bioremediation is encouraging them to work by supplying optimum levels of nutrients and other chemicals essential for their metabolism in order to degrade/detoxify substances which is hazardous to environment and living things. All metabolic reactions are mediated by enzymes. These belong to the groups of oxidoreductases, hydrolases, lyases, transferases, isomerases and ligases. Many enzymes have a remarkably wide degradation capacity due to their non-specific and specific substrate affinity. For bioremediation to be effective, microorganisms must enzymatically attack the pollutants and convert them to harmless products. As bioremediation can be effective only where environmental conditions permit microbial growth and activity, its application often involves the manipulation of environmental parameters to allow microbial growth and degradation to proceed at a faster rate [17].

Bioremediation is occurred naturally and encouraged with in addition of living things and fertilizers. Bioremediation technology is principally based on biodegradation. It refer to complete removal of organic toxic pollutants in to harmless or naturally occurring compounds like carbon dioxide, water, inorganic compounds which are safe for human, animal, plant and aquatic life [18]. Numerous mechanisms and pathways have been elucidated for the biodegradation of a wide variety of organic compounds; for instance, it is completed in the presence and absence oxygen.

The advantage of Bioremediation

01. It is a natural process, it takes a little time, as an acceptable waste treatment process for contaminated material such as soil. Microbes able to degrade the contaminant and increase in numbers when the contaminant is present. When the contaminant is degraded, the biodegradative population become declines. The residues for the treatment are usually harmless product including water carbon dioxide and cell biomass.
02. It requires a very less effort and can often be carried out on site, often without causing a major disruption of normal activities. This also eliminates the need to transport quantities of waste off site and the potential threats to human health and the environment that can arise during transportation.
03. It is applied in a cost effective process as it lost less than the other conventional methods (technologies) that are used for clean-up of hazardous waste. Important method for the treatment of oil-contaminated sites [19].
04. It also helps in complete destruction of the pollutants, many of the hazardous compounds can be transformed to harmless products, and this feature also eliminates the chance of future liability associated with treatment and disposal of contaminated material.
05. It does not use any dangerous chemicals. Nutrients especially fertilizers added to make active and fast microbial growth. Commonly, used on lawns and gardens. Because of bioremediation change harmful chemicals into water and harmless gases, the harmful chemicals are completely destroyed [20].
06. Simple, less labor intensive and cheap due to their natural role in the environment.
07. Eco-friendly and sustainable [21].
08. Contaminants are destroyed, not simply transferred to different environmental media.
09. Nonintrusive, potentially allowing for continued site use.
10. Relative ease of implementation [17].
11. Effective way of remediating natural ecosystem from a number contaminate and act as environment friendly options [22].

The disadvantage of Bioremediation

01. It is limited to those compounds that are biodegradable. Not all compounds are susceptible to rapid and complete degradation.
02. There are some concerns that the products of biodegradation may be more persistent or toxic than the parent compound.
03. Biological processes are often highly specific. Important site factors required for success include the presence of metabolically capable microbial populations, suitable environmental growth conditions, and appropriate levels of nutrients and contaminants.
04. It is difficult to extrapolate from bench and pilot-scale studies to full-scale field operations.
05. Research is needed to develop and engineer bioremediation technologies that are appropriate for sites with complex mixtures of contaminants that are not evenly dispersed in the environment. Contaminants may be present as solids, liquids and gases.
06. It often takes longer than other treatment options, such as excavation and removal of soil or incineration.
07. Regulatory uncertainty remains regarding acceptable performance criteria for bioremediation. There is no accepted definition of “clean”, evaluating performance of bioremediation is difficult.

II. Genetically Modified Microorganism

Accumulated toxic and persistent compounds especially xenobiotics in the environment cannot be degraded efficiently by the natural metabolic diversity of the autochthonous microbes. The novel nature of these pollutants which includes unusual chemical bonding or substitutions with halogens or other functional groups makes them resistant to natural degradation by the inhabiting microorganisms (MOs), due to the lack of appropriate metabolic pathway. Hydrophobic nature of various toxic contaminants in environment such as toluene, organochlorides, and polyaromatic hydrocarbons accumulates in the microbial cell and disrupts their cell membrane (Pieper and Reineke 2000). The prolonged persistence of these compounds is due to the inefficient trans- port systems and their limited bioavailability because of their hydrophobic nature. However, the natural inhabiting consortia at various physiological environmental conditions provide the genetic sources of novel metabolic capabilities (Pieper and Reineke 2000) and the possibilities of degrading these recalcitrant chemicals using genetically modified bacterial strains through the introduction of different enzyme activities from a number of bacteria into a desired microorganism (Chen and Mulchandani 1998). Microorganisms have been evolving since last 3.8 billion years and inhabit virtually in all environmental conditions like extremes of nutrient concentration, salinity, pH, pressure, and temperature (DeLong and Pace 2001). During the long evolutionary process, dif- ferent microorganisms gained unique abilities to degrade persistent compounds at contaminated sites by the physiochemical pressure in those environmental conditions. The potential of this natural microflora can be utilized by identifying and engineering catabolic gene pool. The use of indigenous microflora instead of exotic strains is always naturally advantageous for the construction of recombinant microorganisms because the indigenous microorganisms are more prone to interact with total population and can withstand to the complex stressful environmental conditions (Singh et al. 2011). Catechol 1,2-dioxyge- nase, toluene dioxygenase-iron-sulfur protein component, benzene dioxygenase, naphthalene dioxygenase (Moharikar et al. 2003), dioxin dioxygenase of Sphingomonas sp. strain RW1 (Armengaud et al. 1998), carbazole-1,9-dioxyge- nase (Sato et al. 1997), and many ring-activating dioxygenases (Fuenmayor et al. 1998) are some of the examples of novel enzymes, identified for recombinant DNA technology to improve strains for better bioremediation capabilities.

A multidisciplinary involvement of microbiol- ogy, molecular biology, biochemistry, bioinfor- matics, and genomics is required to produce genetically engineered microorganisms (GEMs) to overcome various bottlenecks in the cleanup of contaminated sites. There are very few reports where GEMs have been applied and proven to be more efficient than natural MOs in elimination of recalcitrant compounds under natural conditions (de Lorenzo 2009). However, efforts are made to expand the range of compounds that can be degraded by applying the principles of recombi- nant DNA technology (Keasling and Bang 1998; Timmis and Pieper 1999; Ryu and Nam 2000; Ang et al. 2005; Liu et al. 2006; Kapley and Purohit 2009). For bioremediation purpose, the first test release of genetically engineered micro- organisms was commenced by the US Environmental Protection Agency (EPA) in 1996 (Ryan et al. 2000). The successful application of genetically MOs for bioremediation is based upon the successful establishment of the engineered microorganisms in the environmental conditions and after the completion of the desired objectives; there should be an appropriate mecha- nism for their removal from the site of action (Carlos and Alkorta 1999). The potential risks associate with the release of GEMs into the envi- ronment has originated the necessity to construct the biological containment systems by which bacteria are killed in a controlled suicide process (Carlos and Alkorta 1999).

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SCHOOL OF BIO AND CHEMICAL ENGINEERING

DEPARTMENT OF BIOTECHNOLOGY

UNIT – IV – Basic Industrial Biotechnology – SBB2202

1. Role of Microbes in degradation of Pollutants

Enzymes such as amylases, carboxymethylcellulases and proteases are widely used in the industry for the manufacture of pharmaceuticals, foods, beverages and confection- eries as well as in textile and leather processing, and waste water treatment [1]. The potential applications of amylases [2], cellulases [2] and proteases [3] in biotechnology have already been reviewed. The majority of the enzymes used in the industry are of microbial origin because microbial enzymes are relatively more stable than the corresponding enzymes derived from plants and animals.

With the recent advent of biotechnology, there has been a growing interest and demand for enzymes with novel properties. Considerable efforts have been devoted to the selection of microorganisms via sophisticated screening techniques and process methodology for the production of enzymes with new physiological/physical properties and tolerance to extreme conditions used in the industrial processes (e.g. temperature, salts and pH). The marine environment has proven to be a rich source of both biological and chemical diversity [4]. The potential of marine organisms for commercial development impinges on virtually every area of biotechnology [5]. Marine microorganisms have recently emerged as a rich source for the isolation of industrial enzymes [6]. Marine bacterial enzymes have several advantages for industrial utilisation [7]. The optimum activity of marine bacterial enzymes usually occurs at high salinity, making these enzymes utilisable in many harsh industrial processes, where the concentrated salt solutions used would otherwise inhibit many enzymatic transformations. In addition, most marine bacterial enzymes are considerably thermotolerant, remaining stable at room temperature over long periods.

The microhabitats for marine bacteria are seawater, sediments, animate and inanimate surfaces. Marine sedentary organisms (marine plants and sessile animals) form highly specific and symbiotic relationships with bacteria [8, 9, 10] and in extreme cases up to 60% of the tissue volume [11]. The surfaces and internal spaces of these organisms are more nutrient rich than seawater and most sediments, thus they would likely to be a unique niche for the isolation of diverse assemblages of bacteria. These associated bac- teria are a potential source of biologically active compounds of low molecular weight [12–14]. On the other hand proteins, especially enzymes, have been largely neglected. Few enzymes such as acetylcholinesterase [15], amylase [16], urethanase [17], and cel- lulase, alginate and pectin lyases [18] have been isolated from bacteria and fungi asso- ciated with marine sponges and algae. Whether the associated bacteria are a potential source of enzymes or the concentrations of enzymes are of only minor importance, is unknown.

II. Biopesticides

Bio-pesticides are those biological agents that are used for control of weeds, insects and pathogens.

The micro-organisms used as bio-pesticides are viruses, bacteria, protozoa, fungi and mites. Some of the bio-pesticides are being used on a commercial scale.

Most important example is the soil bacterium, Bacillus thuringiensis (Bt). Spores of this bacterium possess the insecticidal Cry protein.

Therefore, spores of this bacterium kill larvae of certain insects. The commercial preparations of B. thuringiensis contain a mixture of spores, Cry protein and an inert carrier.

This bacterium was the first bio-pesticide to be used on a commercial scale in the world, and is the first bio-pesticide being produced on a commercial scale in India.

Bio-pesticides are of two types:

(i) Bio-herbicides:

Herbicides are chemicals that are used for inhibiting the growth of plants in unwanted places. Herbicides used for controlling weeds in the cultivated areas are called weedicides. A number of risks are involved in the use of chemical herbicides. This can be avoided if herbicide resistance can be introduced in the crop plants. It is possible through genetic engineering or recombinant DNA technology. Transgenic Tomato and Tobacco plants have been developed which show tolerance to specific herbicides.

Certain crop plants do not allow the weeds to grow nearby. They are called smoother crops, e.g., Barley, Rye, Sorghum, Millet, Sweet clover, Alfalfa, Soybean, Sunflower. Smoother crops eliminate weeds through chemicals. Crop rotation with these crops will naturally reduce the incidence of weeds.

Another way of weed control is the introduction of specific insects which feed on the weeds. Extensive growth of Opuntia in India and Australia was checked through the intro¬duction of its natural herbivore, cochineal insect (Cactoblastis cactorum). Similarly, growth of Hypericum perforatum or Klamath weed was checked by U.S.A. through the introduction of Chrysolina beetles.

An organism which controls or destroys unwanted plant growth without harming the useful plant is called bioherbicide. The first bioherbicide happened to be mycoherbicide. It was put to use in 1981. The herbicide is Phytophthora palmivora. The fungus does not allow the Milkweed Vine to grow in Citrus orchards. Growth of Eichhornia crassipes (Water Hyacinth) is being controlled by Cercospora rodmanii in USA and Alternaria eichhorniae in India.

Puccinia chondrilla has controlled the growth of skeleton weed, Chondrilla juncea in Australia. Fungal spores are now available to be sprayed over weeds for their elimination. Two of them are 'Devine' and 'Collego'. The spores are ideal for marketing because they can tolerate adverse conditions and can remain viable for long periods.

(ii) Bio-insecticides:

Bio-insecticides are those biological agents that are used to control harmful insects. They include the following.

(a) Predators:

Destructive insects or plant pests can be brought under control through introduction of their natural predators. The predators should be specific and unable to harm the useful insects. Introduction of ladybugs (Lady Bird Beetles) and Praying Mantis has been successful in combating scale insects or aphids which feed on plant sap.

(b) Parasites and Pathogens:

This is alternate biological control of plant pests through the search of their natural parasites and pathogens. They include viruses, bacteria, fungi and insect parasitoids. Parasitoids are organisms that live as parasites for some time (as early or larval stage) and free living at other times, e.g., Trichogramma. Nucleopolyhedrovirus (NPV) are species specific.

For example, Baculovirus heliothis (a virus) can control Cotton bollworm (Heliothis Zea). Similarly, Bacillus thuringenesis (a bacterium) is effective against the cabbage looper (Trichoplausiani) and Entomophthora ignobilis (a fungus) the green peach aphid of Potato (Myzus persicae). In U.S.S.R. the fungus Beauveria bassiana has been successfully employed in controlling Potato beetle and Codling moth.

(c) Natural Insecticides:

They are insecticides and related pesticides which are ob¬tained from microbes and plants. A number of natural insecticides are available. The common ones include (i) Azadirachtin from Margosa or Neem (Azadirachta indica). It occurs in Margosa extract. Spray of the same keeps away the Japanese beetles and other leaf eating pests because of the antifeedant property of azadirachtin. (ii) Rotenones. They are powerful insecticides which are harmless to warm blooded animals. Chinese are believed to be first to discover their insecticidal properties. Rotenones are obtained from the roots of Derris elliptica and Lonchocarpus nicou. (iii) Squill. The red variety of Sea Onion (Red Squill, Ureginea maritima) produces a radicide which does not have any harmful effect on other animals, (iv) Nicotine. It is obtained from Nicotiana species. The purified chemical is highly poisonous. Nicotine sulphate is one of the most toxic insecticides, (v) Pyrethrum.

It is an insecticide which is obtained from the inflorescence of Chrysanthemum cinerarifolium (Dalmation Pyrethrum), C. coccineum and C. marshallii. The active compounds are pyrethrin and cinerin. Pyrethrin is also used in fly sprays, aerosols, mosquito coils, etc. (vi) Thurioside. It is a toxin produced by bacterium Bacillus thuringenesis. The toxin is highly effective against different groups of insects like moths, flies, mosquitoes and beetles. It does not cause any adverse environmental pollution or disturbance.

Thurioside occurs as crystals in the bacterium. It kills the susceptible insects through inhibiting ion transport in the midgut, formation of pores in gut epithelium, swelling and bursting of cells, (vii) Transgenic Plants. They are crop plants which are modified through genetic engineering to develop natural resistance to insects by inserting cry genes of Bacillus thuringenesis into them, e.g., Bt Cotton. Similarly, transgenic Tomato has been developed which is resistant to homworm larvae.

III. Biofertilizers

Chemical fertilizers are being used in increasing amounts in order to increase output in high yielding varieties of crop plants.

However, chemical fertilizers cause pollution of water bodies as well as ground water, besides getting stored in crop plants.

Therefore, environmen¬talists are pressing for switch over to organic farming.

Organic farming is the raising of unpolluted crops through the use of manures, bifertilizers and biopesticides that provide optimum nutrients to crop plants, keeping pests and pathogens under control.

Bio-fertilizers are micro-organisms which bring about nutrient enrichment of soil by enhancing the availability of nutrients to crops. The micro-organisms which act as bio-fertilizers are bacteria, cyanobacteria (blue green algae) and mycorrhizal fungi. Bacteria and cynobacteria have the property of nitrogen fixation while mycorrhizal fungi preferentially withdraw min¬erals from organic matter for the plant with which they are associated.

Nitrogen fixation is the process of conversion of molecular or dinitrogen into nitrogen compounds. Insoluble forms of soil phosphorus are converted into soluble forms by certain micro-organisms. This makes the phosphorus available to the plants. Phosphate is also solubilised by some bacteria and by some fungi that form association with plant roots.

The various bio-fertilizers are as follows.

(i) Free Living Nitrogen Fixing Bacteria:

They live freely in the soil and perform nitrogen fixation. Some of them are saprotrophic, living on organic remains, e.g., Azoto- bacter, Bacillus polymyxa, Clostridium, Beijerinckia. They are further distinguished into aerobic and anaerobic forms.

The property of nitrogen fixation is also found in photoautotrophic bacteria, e.g., Rhodopseudomonas, Rhodospirillum, Chromatium. Inoculation of soil with these bacteria helps in increasing yield and saving of nitrogen fertilizers. For example, Azotobacter occurring in fields of

Cotton, Maize, Jowar and Rice, not only in¬creases yield but also saves nitrogen fertilizer to the tune of 10-25 kg/ha. Its inoculation is available under the trade name of azotobactrin.

(ii) Free Living Nitrogen Fixing Cyanobacteria:

A number of free living cyanobacteria or blue-green algae have the property of nitrogen fixation, e.g., Anabaena, Nostoc, Aulosira, Totypothrix, Cylindrospermum, Stigonema. Cyanobacteria are photosynthetic. Therefore, they add organic matter as well as extra nitrogen to the soil.

Aulosira fertilissima is consid¬ered to be the most active nitrogen fixer of Rice fields in India (Aiyer et al, 1972). Cylindrospermum licheniforme grows in Sugarcane and Maize fields. Cyanobacteria are an extremely low cost biofertilisers. In Tamil Nadu, the technique of cyanobacteria inoculation to rice fields is being followed. Phosphate, Molybdenum and Potassium are supplied addi¬tionally.

(iii) Loose Association of Nitrogen Fixing Bacteria:

Certain nitrogen fixing bacteria like Azospirillum live around the roots of higher plants without developing any intimate relationship. It is often called rhizosphere association. The bacteria obtain some plant exudate and use the same as part of their food requirement. The bacteria fix nitrogen and exude a part of the fixed nitrogen for use by the plant. The phenomenon is termed as associative mutualism (= associative symbiosis).

(iv) Symbiotic Nitrogen Fixing Bacteria:

They form a mutually beneficial association with the plants. The bacteria obtain food and shelter from plants. In return, they give a part of their fixed nitrogen to the plants. The most important of the symbiotic nitrogen fixing bacteria is Rhizobium (pi Rhizobia). It forms nodules on the roots of legume plants. There are about a dozen species of Rhizobium which form association with different legume roots, e.g., R. leguminosarum, R. lupini, R. trifolii, R. meliloti, R. phaseoli.

These bacteria, also called rhizobia, live freely in the soil but cannot fix nitrogen except for a strain of Cowpea Rhizobium (Me Comb et al, 1975). They develop the ability to fix nitrogen only when they are present inside the root nodules. In the nodule cells, bacteria (bacteroids) lie in groups

surrounded by membrane of the host which is lined by a pink-red pigment called leghaemoglobin. Presently cultures of Rhizobium specific for different crops are raised in the laboratory.

Frankia, a nitrogen fixing mycelial bacterium (actinomycete), is associated symbiotically with the root nodules of several nonlegume plants like Casuarina, Alnus (Alder) Myrica, Rubus etc. Leaves of a few plants (e.g., Ardisia) develop special internal cavities for pro-viding space to symbiotic nitrogen fixing bacteria, Xanthomonas and Mycobacterium. Such leaves are a constant source of nitrogen fertilizer to the soil.

(v) Symbiotic Nitrogen Fixing Cyanobacteria:

Nitrogen fixing cyanobacteria (blue- green algae) form symbiotic association with several plants, e.g., cycad roots, lichens, liverworts, Azolla (fern). Out of these, Azolla-Anabaena association is of great importance to agriculture.

Azolla pinnata is a small free floating fresh water fern which multiplies rapidly, doubling every 5-7 days. The fern can coexist with rice plants because it does not interfere with their growth. In some South-East Asian countries, especially China, the rice fields are regularly provided with Azolla.

Anabaena azollae resides in the leaf cavities of the fern. It fixes nitrogen. A part of the fixed nitrogen is excreted in the cavities and becomes available to the fern. The decaying fern plants release the same for utilization of the rice plants. When field is dried at the time of harvesting, the fern functions as the green manure, decomposing and enriching the field for the next crop.

(vi) Microphos Biofertilizers:

They release phosphate from bound and insoluble states, e.g., Bacillus polymyxa, Pseudomonas striata, Aspergillus species.

(vii) Mycorrhiza (pl-Mycorrhizae Frank,. 1885):

It is a mutually beneficial or symbiotic association of a fungus with the root of a higher plant. The most common fungal partners of mycorrhiza are Glomus species. Mycorrhizal roots show a sparse or dense wooly growth of fungal hyphae on their surface. Root cap and root hairs are absent.

The shape is irregular, tuberous, nodulated or coralloid. The fungus remains restricted to the cortex of the root. The vascular strand and growing point are not affected. Mycorrhiza often remains in the upper layers of the soil where organic matter is abundant. Depending upon the residence of the fungus, mycorrhizae are of two types— ectomycorrhiza and endomycorrhiza.

(a) Ectomycorrhiza (= Ectotrophic Mycorrhiza):

The fungus forms a mantle on the surface of the root. Internally, it lies in the intercellular spaces of the cortex. The root cells secrete sugars and other food ingredients into the intercellular spaces for feeding the fungal hyphae. The exposed fungal hyphae increase the surface of the root to several times. They perform several functions for the plant—

:(i) Absorption of water,

(ii) Solubilisation of organic matter of the soil humus, release of inorganic nutrients, absorption and their transfer to root,

(iii) Direct absorption of minerals from the soil over a large area and handing over the same to the root. Plants with ectomycorrhiza are known to absorb 2-3 times more of nitrogen, phosphorus, potassium and calcium,

(iv) The fungus secretes antimicrobial sub¬stances which protect the young roots from attack of pathogens. Ectomycorrhiza occurs in the trees like Eucalyptus, Oak (Quercus), Peach, Pine, etc. The fungus partner is generally specific. It belongs to basidiomycetes.

(b) Endomycorrhiza (- Endotrophic Mycorrhiza):

Fewer fungal hyphae lie on the surface. The remaining live in the cortex of the root, mostly in the intercellular spaces with some hyphal tips passing inside the cortical cells, e.g., grasses, crop plants, orchids and some woody plants. In seedling stage of orchids, the fungal hyphae also provide nourishment by forming nutrients rich cells called pelotons. Intracellular growth occurs in order to obtain nourishment because unlike ectomycorrhiza, the cortical cells do not secrete sugars in the intercellular spaces.

The hyphal tips passing into cortical cells either produce swollen vesicles or finely branched masses called arbuscules. Therefore, endomycorrhiza is also called VAM or vesicular-arbuscular

mycorrhiza. The major benefits of VAM to the plant are the supply of inorganic nutrients as well as enhanced water absorption. Phosphate which is mostly present in the unavailable form in the soil, becomes abundantly available to the plant. A single fungus may form mycorrhizal association with a number of plants, e.g., Glomus.

Importance of Bio-fertilizers:

(i) They increase the yield of plants by 15-35%.

(ii) Bio-fertilizers are effective even under semi-arid conditions,

(iii) Farmers can prepare the inoculum themselves,

(iv) They improve soil texture,

(v) Bio-fertilizers do not allow pathogens to flourish,

(vi) They produce vitamins and growth promoting bio-chemical’s,

(vii) They are non-polluting.

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SCHOOL OF BIO AND CHEMICAL ENGINEERING

DEPARTMENT OF BIOTECHNOLOGY

UNIT –V – Basic Industrial Biotechnology – SBB2202

1. Vaccines

01. Several genes from different etiologic agents have been cloned, expressed and purified to be tested as vaccines. There are a variety of expression systems for antigenic protein components, such as bacteria, yeast, mammalian cells and insect cells, in which the DNA encoding the antigenic determinant can be inserted and expressed. However, several factors must be taken into account before selecting the system for antigen expression.

02. The level of expression obtained using each specific expression vector and promoter, the selection marker of choice, the presence or absence of post-translational modification by the recombinant vector, among others, are essential features that interfere in the efficacy of production of recombinant antigens as vaccines. Bacterial expression systems are the most used due to the ease of handling and to their capacity for high level expression. However, for antigens in which post-translational modifications (e.g., glycosylation) are necessary, the use of mammalian or insect cells should be considered.

03. Recombinant protein vaccines:

    1. Most of the vaccines under investigation today are based on highly purified recombinant proteins or subunits of pathogens. The classical example of recombinant protein vaccines currently in use in humans is the vaccine against hepatitis B (Table 1). Hepatitis B virus (HBV) infection is a chronic liver disease occurring worldwide.

    2. HBV presents a marked tropism for human liver cells, partially due to a specific receptor that is expressed on the surface of infected cells. The current vaccines are produced by expressing the hepatitis B surface antigen (HBsAg) in yeast cells. The HBsAg assembles into virus-like particles (VLPs), which are extremely immunogenic, making the HBV vaccine a very efficacious vaccine. The yeast expression system may secrete the antigen into the culture supernatant that can facilitate its purification.

    3. Furthermore, yeast cells offer some of the eukaryotic cellular machinery responsible for the post-translational modification of proteins, being capable of rendering proteins glycosylated. The technology of production of the HBV vaccine has been transferred to several

manufacturers and the prices have decreased due to competition, which has rendered this vaccine affordable to most developing countries.

Table 1.
Licensed[a] viral and bacterial vaccines for use in humans.

	Live attenuated	Killed inactivated	Subunit
Viral	Vaccinia	Polio (IPV)	Hepatitis B (HepB-surface antigen)
	Polio (OPV)	Rabies	Human papilloma virus (HPV)
	Yellow fever	Influenza	
	Measles	Hepatitis A	
	Mumps		
	Rubella		
	Influenza		
	Rotavirus		
Bacterial	BCG (tuberculosis)	*Bordetella pertussis* (whole cell)	Tetanus (toxoid)
	Salmonella typhi (oral)	Cholera	Diphtheria (toxoid)
		Bacillus anthracis	*Neisseria meningitidis* (polysaccharide)
			Bordetella pertussis (acellular)
			Streptococcus pneumoniae, 23 valent (polysaccharide)
			Haemophilus influenzae, type b (Hib) (polysaccharide)
			Neisseria meningitidis (polysaccharide conjugate)
			Streptococcus pneumoniae, heptavalent (conjugate polysaccharides)
			Salmonella typhi Vi (capsular polysaccharide)

[a]Licensed by national regulatory agencies such as ANVISA in Brazil or FDA in the USA.
OPV = oral polio vaccine; IPV = inactivated polio vaccine; BCG = bacillus Calmette-Guérin.

04. Live recombinant vaccines using bacterial or viral vectors:

1. As a result of advances in the fields of molecular biology and genetic engineering it is now possible to create live recombinant vectors capable of delivering heterologous antigens by the introduction of antigen-encoding genes. The idea behind this approach is to use the capacity of infection and the immunological properties of the live vector to elicit an immune response against its own proteins, as well as towards the heterologous protein being presented.

2. A number of bacteria [such as *Salmonella typhi* and bacille Calmette-Guérin (BCG)] and viruses [such as vaccinia (smallpox) and adenovirus] have been investigated as live recombinant vector vaccines. In general, these approaches have advantages that are intrinsic to the pathogen itself, such as mimicry of a natural infection, their capacity of stimulating

both $CD4^+$ and $CD8^+$ T-cell subsets, and, in some cases, the possibility to be administered orally.

3. The use of live-attenuated bacterial vaccines is not novel. However, their utilization as carriers or delivery vehicles for heterologous antigen expression represents a technology with broad applicability that may have a significant impact on vaccine development. Significant advances in molecular biology have enabled precise deletions of genes encoding important virulence factors, as well as the introduction of recombinant DNA into avirulent yet immunogenic vaccine strains.

4. Bacterial vectors have many advantages that make them attractive systems for heterologous antigen presentation. They can elicit humoral and/or cellular immune responses and can be administered orally, thereby eliciting mucosal immunity. Most are antibiotic-sensitive strains, which allow antibiotic treatment if any adverse reaction occurs. In general, they display very favorable cost-effectiveness.

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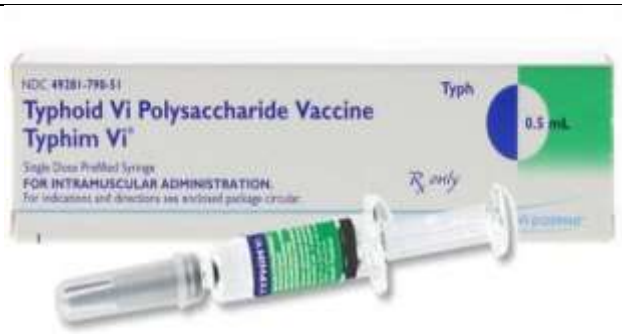
<td> 
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<td>Vaccinia (Smallpox) vaccine</td>
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05. DNA Vaccines

1. DNA vaccination is a technique for protecting an organism against disease by injecting it with genetically engineered DNA to produce an immunological response. Nucleic acid vaccines are still experimental, and have been applied to a number of viral, bacterial and parasitic models of disease, as well as to several tumour models. DNA vaccines have a number of advantages over conventional vaccines, including the ability to induce a wider range of immune response types.

2. Vaccines are among the greatest achievements of modern medicine – in industrial nations, they have eliminated naturally-occurring cases of smallpox, and nearly eliminated polio, while other diseases, such as typhus, rotavirus, hepatitis A and B and others are well controlled. Conventional vaccines, however, only cover a small number of diseases, and infections that lack effective vaccines kill millions of people every year, with AIDS, hepatitis C and malaria being particularly common.

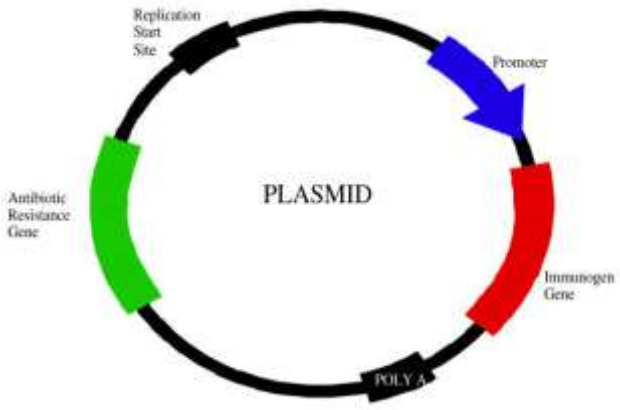
3. The vaccine DNA is injected into the cells of the body, where the "inner machinery" of the host cells "reads" the DNA and converts it into pathogenic proteins. Because these proteins are recognised as foreign, they are processed by the host cells and displayed on their surface, to alert the immune system, which then triggers a range of immune responses. These DNA vaccines developed from “failed” gene therapy experiments.

4. A DNA vaccine (or genetic vaccine as it is also called) consists of a plasmid containing:

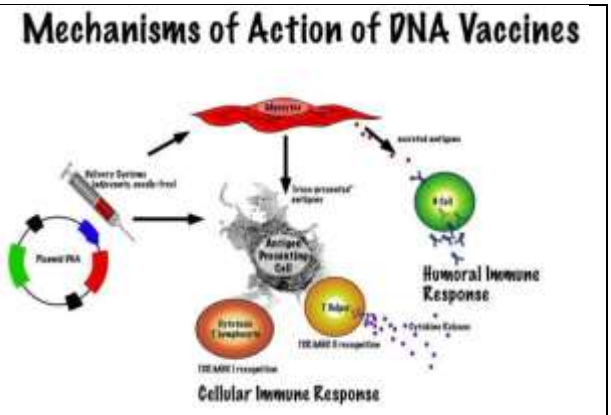
    a. One origin of replication of *Escherichia coli*, for the amplification of the plasmid
    b. A strong promoter, generally from cytomegalovirus
    c. Multiple Cloning Sites, in which one can insert the gene to be expressed
    d. An antibiotic as selection marker

5. The first demonstration of a plasmid-induced immune response was when mice inoculated with a plasmid expressing human growth hormone elicited antibodies instead of altering growth. Thus far, few experimental trials have evoked a response sufficiently strong enough to protect against disease, and the usefulness of the technique, while tantalizing, remains to be conclusively proven in human trials. However, in June 2006 positive results were

announced for a bird flu DNA vaccine and a veterinary DNA vaccine to protect horses from West Nile virus has been approved In August 2007, a preliminary study in DNA vaccination against multiple sclerosis was reported as being effective.



Components of DNA Vaccines



BCG Vaccine

6. Advantages and disadvantages of DNA vaccines are listed in the following slide

Advantages	Disadvantages
• Subunit vaccination with no risk for infection • Antigen presentation by both MHC class I and class II molecules • Able to polarise T-cell help toward type 1 or type 2 • Immune response focused only on antigen of interest • Ease of development and production • Stability of vaccine for storage and shipping • Cost-effectiveness • Obviates need for peptide synthesis, expression and purification of recombinant proteins and the use of toxic adjuvants ,Long-term persistence of immunogen • In vivo expression ensures protein more closely resembles normal eukaryotic structure, with accompanying post-translational modifications	• Limited to protein immunogens • Potential for atypical processing of bacterial and parasite proteins

II. Bioprocess strategies in Plant Cell and Animal Cell culture

Plants have evolved a vast chemical cornucopia driven primarily by the requirement to protect themselves from microbial pathogens, insect pests and grazing animals. To date approximately 200,000 NPs have been identified (1) and some of these have been utilised as drugs (codeine, morphine, paclitaxel) (2), food flavouring and colouring agents (saffron, spearmint and anthocyanins) (3, 4), pest and disease management chemicals (nicotine, strychnine and azadirachtin) (5, 6) and cosmetics and fragrance products (lavender, rosemary and Aloe vera) (7, 8). However, only a tiny fraction of this vast chemical space has been mined to date, therefore many blockbuster molecules await exploitation.

A variety of different methods have been developed to produce NPs, depending on molecular complexity, relative demand and the economics of production. A common strategy for obtaining NPs is extraction from the source plant but typically these plants have not been domesticated and their wild-populations maybe limiting. Additionally, the slow growth rate of the given source plant frequently reduces production potential. The typically low concentration of the active molecule in the source plant is also a potential brake on production.

The total chemical synthesis of NPs is an important alternative strategy. For molecules of relatively simple structure this provides an attractive route for production. Many NPs, however, have multiple chiral centres with region-specific and stereo-specific properties associated to their function (9), making total chemical synthesis either difficult or unprofitable. The production of NPs via semi-synthesis represents another approach that can circumvent some of the issues associated with the total synthesis of these high-value plant chemicals. This strategy is based on harvesting a more abundant chemical precursor integral to the biosynthetic pathway responsible for the generation of the target NP. For example, the production of the diterpenoid, paclitaxel (10). Semi-synthesis routes, however, still typically remain costly and often also generate toxic by-products, which can be damaging to the environment (9).

Plant cell culture (PCC) is a well-established technology platform for the synthesis of NPs. This approach provides several advantages in comparison to other potential strategies, especially for the production of NPs with complex structures. Significant progress has been made recently in plant cells platforms, building on the established expertise in microbial and animal cell culture. This has enabled the successful scale-up from experimental platforms to commercially feasible industrial

scale processes. Thus, facilitating a series of high-value NPs to become commercially competitive in the market place, especially those of high-value (11).

The development of powerful new "omics" technologies, including next generation sequencing has provided exciting opportunities for PCC. In this context, the number of patents related to PCC products has risen to a total of 28,000 and the amount of companies utilising PCC or hairy root technologies for the production of NPs associated with the cosmetic, food and pharmaceutical industries continues to rapidly expand (Table 1). Here we provide an update on the generation of NPs utilising PCC and highlight some of the strategies that can be undertaken to increase NP yields including: cell line selection, permeabilisation, biotransformation, scale-up strategies, immobilization, product secretion; and, the opportunities presented by the relatively recent isolation and culture of cambial meristematic cells (CMCs).

Table 1.

Commercial production of natural products and heterologous proteins in plant suspension and hairy root culture. Modified from (15)

Industry	Products	Species	Manufacturer	Use/Notes
Plant Natural Products				
Food	Anthocyanins	*Euphorbio milii*	Nippon Paint Co. Ltd, Osaka Japan	Textile dye
		Aralia cordata		Coloring agents

	Arbtin	*Catharanthus roseus*	Mitsui Chemicals Inc., Tokio Japan	Pigments
	Betacyanin	*Beta vulgaris*	Nippon Shinyaku Co., Ltd	Pigments
	Carthamin	*Carthamus trinctorius*	Kibun Foods Inc., Tokio Japan	Pigments
	Geraniol	*Geraminea spp.*	Mitsui chemicals., Inc	Essential Oils
	Gingseng	*Panax gingseng*	Nitto Denko Corporation, Osaka, Japan	Dietary supplements
		Wild gingseng from CMCs	Unhwa Biotech Corp., Jeonbuk, South Korea	Dietary supplements, cosmetic and medical products
	Shikonin	*Lithospemum erythrorhizon*	Mitsui Chemicals., Inc	Red pigments
Pharmaceuticals	Berberines	*Coptis japonica*	Mitsui	Anticancer

			Chemicals, Inc	
		Thalictrum minus		Antibiotic Anti-inflammatory
	Echinacea polysaccharides	*Echinacea purpurea*	Diversa, Ahrensburg, Germany	Immunostimulant
		Echinacea angustifolia		Anti-inflammatory
	Paclitaxel	Taxus spp	Phyton Biotech., Inc Germany	Anticancer World
			Samyang Genex., Seoul, South Korea	Anticancer
	Podophyllotoxin	Podophyllum spp.	Nippon Oil, Tokio, Japan	Anticancer
	Rosamarinic acid	*Coleus blumei*	ANattermann & Cie. Gmbh, Cologne, Germany	Anti-inflammatory

	Scopolamine	Duboisia spp.	Sumitomo Chemical Co., Ltd, Tokio, Japan	Anticancer World
				Antimuscrnic
				Used in treatment of motion sickness, nausea and intestinal cramping
Cosmetic	Atropine, Gingsenosides, Coumarines, Flavonoids, Alkaloids, Camptothechin, Anabasine, Nicotine	Hairy roots from *Atropa belladonna Carlina acaulis Nicotiana glauca Panax gingsen*	Rootec, Witterswil, Switzerland	A wide range of products for beauty health and nutrition
	About fifteen active ingredients for cosmetic use	Cell suspension cultures and hairy roots cultures, different species	Sederma, Le Perray-en-Yvelines, France	Develop of cosmetic active ingredients
	Active ingredients for cosmetic use	Suspension culture and hairy roots *Malus domestica Solar*	Mibelle, Switzerland	Develop of cosmetic active ingredi

		vitis		
	Human Glucocerebrosidase (GCD) enzyme	Carrot suspension cultures	Protalix BioTherapeutics Karmiel, Israel	ELELYSO® Plant cell-expressed form of the glucocerebrosidase enzyme for treatment of Gaucher disease
	Vaccine against Newcastle disease virus (NDV)	Tobacco suspension cultures	Dow Agrosciences, LLC, Indianapolis, USA	First tobacco cell-based vaccine approved by the FDA against Newcastle disease virus in poultry
	Human proteins	*Physcomitrella patens* suspension cultures	Greenovation Biotech BMBH Freiburg, Germany	α-galactosidase for Fabry disease and βα for Gaucher disease.
				Both are in phase 1 of clinical trials
	Glycosylated recombinant proteins	Hairy roots	Root Lines	Rhizo prot platform
			Amiens, France	

PLANT CELL CULTURES

PCC has become a well establish platform to produce plant NPs for the food, cosmetic and drug industries. In addition, this platform provides important tools to drive basic research. The production of valuable NPs using PCC has begun to garner significant attention due to its advantages relative to the production of NPs from natural harvest or semi-synthesis, from more abundant natural precursors (12). PCC provides a powerful production system for NPs independent from constraints resulting form geographical and seasonal variation and also limitations resulting from access to often dwindling wild plant populations. Thus, the PCC platform can aid the establishment of robust supply chains and help alleviate ecological problems and the loss of plant biodiversity (13-15). Importantly, the production of NPs from PCC is independent of food production. Further, the application of PCC technology reduces the requirement for precious water resources associated with contemporary agricultural production regimes. Commercialization of NPs produced via PCC have also gained significant consumer acceptance, since they are categorized as non-GMO (16, 11).

Plant cell suspension cultures offer the most reliable and productive system to generate NPs. An example of this is the production of geraniol, an important monoterpenoid for flavour and fragrance industries (17). While this NP was synthesized to slightly higher levels in transgenic tobacco plants, PCC proved to be overall the more commercially viable system when the simplicity in handling and the continuous nature of the process were taken into consideration. Thus, PCC was established as the ideal plant-based expression platform for the production of geraniol and related NPs (18). Furthermore, by utilising an appropriate culture medium and optimal levels of phytohormones, it is possible to establish in vitro cultures of most plant species. Following successful callus formation, cell suspension cultures can subsequently be generated, simply by adding these cells to a liquid medium. The resulting cultures typically have significant scale-up capability for their growth within industrially relevant bioreactors designed to maximize levels of NP biosynthesis.

HAIRY ROOT CULTURES

The cultivation of differentiated cells in organized tissues such as roots or shoots, constitutes an important alternative strategy for the production of high-value NPs (19). Hairy root culture are generated following infection with the gram-negative, soil bacterium Agrobacterium rhizogenes, which transfers a Transfer (T)-DNA from its large root-inducing (Ri) plasmid into the genome of the infected plant (20). The T-DNA carries a set of genes that encode for enzymes responsible for modulating auxin and cytokinin accumulation. The new hormone balance at the infection site mitotically activates surrounding cells inducing the formation of proliferating roots, so-called hairy roots. This phenotype is characterized by extensive branching, lack of geotropism and a high grow rate in the absence of exogenous hormones, (20, 21). Hairy root cultures are relatively easy to maintain and are genetically stable. Hairy root cultures have a successful track record in the production of a wide range of NPs including nicotine, ginsenosides, camptochecin, tropane and pyrrolizidine alkaloids (19, 20, 22). However, an important limitation in the production of NPs from hairy roots is that the target molecule must be synthesized within the roots of the given source plant. This is significant because relatively few NPs are synthetized in root tissues, with the majority produced in aerial structures. Further, another potential issue is that some root synthesized NPs are often translocated away from their site of production via either the phloem or xylem to distant sink tissues, where they are stored for chemical defence (22). Nevertheless, a number of NPs are both synthesized and accumulated in roots, including: scopolamine, atropine, anthraquinone, indole alkaloids cardiac glycosides (23).

Another major limitation for the industrial-scale production of NPs using hairy roots is the development of appropriate bioreactor production platforms. Recent developments in bioprocess engineering have, nonetheless, driven the construction of bioreactors compatible with the industrial scale-up of hairy root systems. For example, ROOTec has developed a mistbased bioreactor for the production of a number of high-value NPs from a variety of plant species (http://www.rootec.com/) Table 1. In addition to NPs, hairy root cultures have gained attention for the production of pharmaceutical proteins due to their simple growth media requirements, genetic stability and capacity for scale-up (20). Thus, Root Lines Technology Inc. (http://www.rootlines-tech.com/) (Table 1) have exploited these properties for the industrial-scale production of recombinant glycosylated proteins.

CELL LINE SELECTION FOR ENHANCED NP PRODUCTION

Due to the heterogeneous population of typical dedifferentiated cells (DCCs) that comprise callus, the selection of a highly productive cell line has been an essential step for the establishment of profitable production platforms for NPs. As the accumulation of NPs in plants is genotype specific, the selection of suitable species and subsequently organs for callus generation is crucial (24). In addition, this selection process can be facilitated by elegant chemical-based approaches. For example, the identification of cell lines exhibiting a high level of metabolic flux through the targeted pathway by the exogenous application of an intermediate. Thus, cell lines with enhanced biosynthesis of rosmarinic acid in Lavandula vera were obtained by adding two different analogues of phenylalanine (m- and p-F-D,L phenylalanine), the substrate of phenylalanine ammonia-lyase (PAL), to the medium. Only cell lines with high levels of PAL activity were resistant to the analogue, reflecting their ability to produce elevated levels of the target NP, rosmarinic acid (25). In a similar fashion, high producing shoots of Mentha arvenis were screened by the addition of menthol into the medium. The selected clones exhibiting high menthol tolerance identified genotypes with superior performance for the production of increased quantities of menthol, for possible large scale production (26).

A major limitation following the selection of a suitable cell line for NP production is that cell lines often loose their ability to produce the desired NP (15, 19, 27). This decrease or complete loss of NP biosynthesis is thought to be due to genetic instability resulting from somaclonal variation. This chromosomal rearrangement that occurs during long-term subculture, has been associated with aneuploidy and polyploidy. The resulting genetic variation affects chromosome structure causing genetic modifications including deletions and insertions that may affect the production of NPs (28). Subpopulations of cells with different DNA contents due to a change in ploidy were found in Taxus media cells after 1 year of subculture (29). Further, differences in ploidy are related to changes in gene expression, in general higher ploidy appears to result in the silencing of an increased number of genes (28). Changes in culture performance resulting from these deleterious genetic changes typically necessitates periodic screening in order to maintain the desirable characteristics of the given culture system (29). In this context, the application of a selectable marker within plant cells under culture, combined with flow sorting techniques, has been utilised to circumvent this problem, maintaining high performing cells for more than 12 months (30).

CULTURE CONDITION OPTIMIZATION

Following selection of high performing cell lines, another key consideration is to establish optimum media and culture composition. Optimization of growth, maintenance and production media are necessary to achieve high NP production. Although standard PCC medium such as MS (31), SH (32), Gamborg B5 (33) and LS (34) provide a platform for the growth and maintenance of plant cell lines, enhanced production of NPs can be manipulated by further media modification. Significant benefits can be gained by identifying the optimum culture medium environments early in the production timeline, addressing the integrity and stability of the specific NP to be produced. Some of the most useful modification made in the growth medium to promote NP production are related to the source of energy, nitrogen, phosphate, growth regulators, and inoculum density (24).

In general plant cell growth and the production of NPs are inversely related. Thus, production of NPs typically occurs in late stationary phase and is associated with growth inhibition and the production of enzymes for secondary metabolism. Therefore, a dual system to produce NPs is particularly effective, in which the first phase is for cell growth, followed by cell transfer to production medium, which does not support growth, but is favorable for NP biosynthesis (35). Typical modifications to the production medium include the reduction or elimination of phytohormones such as 2,4-D; reduction in phosphate levels; an increase in sucrose levels or modification of the carbohydrate:nitrogen ratio (35).

Screening of high producing genotypes, selection of adequate medium, and optimization of the culture environment for PCC may increase production of NPs by 20-30-fold (19). However, the optimization of plant-based production systems is a relatively nascent area. In this context, promising new strategies are emerging that hold significant promise for future application. For example, the use of a non-invasive, in-line system to monitor cell biomass during culture can be an important step to maximize product yield and quality, while improving culture nutrients (36). Also, culture parameters including levels of phytohormones and components within the media can be optimized more rapidly by employing a statistical design of experiments approach (37). This strategy has been shown to be successful for the optimization of culture parameters for M12 antibody production in hairy root cultures (Häkkinen et al., 2014) and in the production of a human antibody in a BY-2 tobacco cell suspension culture (37). The same strategy has also been used to promote biomass accumulation and geraniol production in tobacco cell suspension cultures (39). Therefore, experimental design strategies, in combination with in-line monitoring, maybe greatly

beneficial during the early steps in screening and optimization for the establishment of high producing cell lines.

ELICITATION

The biosynthesis of NPs is integral to plant immune responses activated in response to attempted pathogens ingress or exposure to a range of abiotic stresses (40). Therefore, so-called elicitors, derived from pathogen associated, plant immune-related or stress signalling molecules, can trigger immune or stress-related NP production. A diverse range of biotic elicitors have been utilized including plant cell wall fragments (pectin and cellulose) and polysaccharides from microorganism (chitin and glucan) (41, 42), plant immune signalling molecules (salicylic acid and methyl-jasmonate (MeJA) (43, 44). Abiotic elicitors include: inorganic salts, heavy metals, UV irradiation, high salinity and high pressure (45). The application of the immune-related MeJA has been particularly widely utilized. For example, addition of MeJA to Taxus cuspidata CMCs induced the production of the blockbuster anticancer drug, paclitaxel, 14,000% in comparison with mock-treated cells (46).

Elicitor concentration, cell age and the stage of the given culture at the moment of elicitation are all important factors necessary to optimize the production of NPs (24). Thus, a significantly higher production of paclitaxel was obtained when MeJA was added to an 8 day-old culture in comparison to a 14 day-old culture (44). The addition of a combination of elicitors is another common practice in PCC with the advantage not only in increasing the production of a given NP but also often its release into the medium. The employment of MeJA in combination with cyclodextrin led to a synergistic effect on resveratrol production in grapevine cell cultures (47). A synergistic effect was also found on paclitaxel accumulation in T. media cells using MeJA and cyclodextrins. Both elicitors induced taxanes and advantageously, paclitaxel was detected mainly in the culture medium. Interestingly, this correlated with elevated expression levels of genes encoding ABC transporters. Therefore, if this process could be successfully scaled-up, purification costs for paclitaxel could be significantly reduced (48).

IMMOBILIZATION OF PLANT CELLS

Immobilization techniques were initially developed to immobilize enzymes for applications in the industrial production of sugars, amino acids and pharmaceutical products (49). Subsequently,

immobilization was utilized to enclose cells. The advantages of this technique are that it provides high cell concentrations for use in small bioreactors, protection from shear stress for cell lines with high sensitivity and increases product formation by enabling maintenance of biomass over a prolonged period (24). Given that most NPs are produced in the stationary phase of growing cells, immobilization potentially could create an excellent environment for the production and accumulation of plant NPs. The most common immobilization agent is calcium alginate which has been deployed in the production of paclitaxel (50), vanillin, ajmalicine and capsaicin (51). However, there are no currently registered companies that utilize plant cell immobilization as a platform to produce NPs. Immobilization has been employed for the commercial production of pharmaceutical proteins where cell encapsulation in alginate increased the production of human granulocyte-macrophage colony-stimulating factor (GM-CSF) in tobacco cells by ~50% (52). Thus, the application of immobilized plant cells may constitute a significant opportunity to enhance the future industrial production of NPs. Nevertheless, limitations associated with nutrient and metabolite mass transfer remain a challenge to be addressed before the implementation of immobilized plant cells at commercial level for NP production can commence.

PERMEABILISATION, SECRETION AND EXTRACTION OF NPs

Permeabilisation aids the release of given products without affecting cell viability and the biosynthesis capacity of cell cultures. Cell permeabilisation facilitates the removal of NPs from vacuoles and membrane systems of the plant cell facilitating the secretion of products into the culture medium, simplifying purification procedures (51). Different permeabilising agents have been used to facilitate the release of the product stored, including the organic solvents isopropanol and dimethylsulfoxide (DMSO) and also chitosan (53). The concentration and time of addition of the permeabilising agent to the medium are key parameters to avoid cell growth inhibition. In the case of Taxus chinensis cell cultures the addition of a low concentration of hexadecane, decanol and dibutylphthalate contributed to the release of paclitaxel into the medium (54).

The accumulation of NPs can be affected by enzymatic or non-enzymatic degradation of the NP in the medium (51) and/or by feedback inhibition of product synthesis (54). The application of in situ product removal, aims to prevent any subsequent cross interference between the product and the producing cell (55). In situ product removal involves direct separation of the product from the culture medium while its production is progressing and may be undertaken either by liquid-solid (in

situ adsorption) or liquid-liquid (in situ extraction) systems (56). These processes can aid product recovery, increasing culture productivity and mitigating toxicity caused by waste materials released from cells, significantly decreasing the cost of downstream processing. Solid-liquid systems such as activated charcoal, XAD-4 and XAD-7 resins typically enable better outcomes compared with liquid-liquid systems. Activated charcoal has been used to increase the production of vanilla and coniferyl aldehyde. XAD-4 has been successful employed for chlorogenic acid, caffeine, paclitaxel and vanilla. While XAD-7, has improved the production of anthraquinonces, ajmalicine, serpentine, plumbagin and paclitaxel (56).

BIOTRANSFORMATION

Biotransformation is another strategy that can be used to produce high-value-chemical products in plant cells or organ tissues (24, 57). Biotransformation involves the chemical conversion of supplied substances catalyzed by biological systems, plant cells organ tissues or enzymes (58, 59). This strategy has the advantage of transforming inexpensive, available and plentiful products into rare, expensive and high-value NPs. The method is based on exploiting enzyme reactions including: hydroxylations, glycosylations, oxidoreduction, hydrogenation, hydrolysis, methylations, acetylation, isomerizations and esterifications (59, 60).

Previously, the application of biotransformation was limited by the absence of knowledge related to plant secondary metabolism (59). However, the recent advances in “omics” technologies have enabled significant advances in our understanding of plant secondary metabolism. Hairy root cultures, due to their genetic and biochemical stability, plant hormone independence and inexpensive culture requirements, offer a particularly attractive platform for the application of biotransformation (58). In this context, biotransformation of cinnamyl alcohol to rosavins in hairy roots of Rhodiola kirilowii produced 80-95% of the glycosides released to the culture media (61). Importantly, biotransformation can drive the discovery of novel plant NPs. For example, a novel terpenoid indole alkaloid in suspension cultures of C. roseus was identified by biotransformation of catharantine. The new metabolite was identified as 3-hydroxy-4-imino-catharanthine (62). Therefore, biotransformation constitutes an important alternative to the semi-synthesis strategy by enabling improved stereospecific characteristics, bioavailabilty and the potential to lower the toxicity of the desired NPs.