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SCHOOL OF BIO AND CHEMICAL ENGINEERING

DEPARTMENT OF BIOTECHNOLOGY

UNIT – I– Bioprocess Engineering -1 – SBT1301

AN INTRODUCTION TO FERMENTATION PROCESSES

The term “fermentation” is derived from the Latin verb *fervere,* to boil, thus describing the appearance of the action of yeast on the extracts of fruit or malted grain. The boiling appearance is due to the production of carbon dioxide bubbles caused by the anaerobic catabolism of the sugar present in the extract. However, fermentation has come to have with different meanings to biochemists and to industrial microbiologists. Its biochemical meaning relates to the generation of energy by the catabolism of organic compounds, whereas its meaning in industrial microbiology tends to be much broader.

The catabolism of sugar is an oxidative process, which results in the production of reduced pyridine nucleotides, which must be reoxidized for the process to continue. Under aerobic conditions, reoxidation of reduced pyridine nucleotide occurs by electron transfer, via the cytochrome system, with oxygen acting as the terminal electron acceptor. However, under anaerobic condition, reduced pyridine nucleotide oxidation is coupled with the reduction of an organic compound, which is often a subsequent product of the catabolic pathway. In the case of the action of yeast on fruit or grain extracts, NADH is regenerated by the reduction of pyruvic acid to ethanol. Different microbial taxa are capable of reducing pyruvate to a wide range of end products, as illustrated in Fig. 1.1. Thus, the term fermentation has been used in a strict biochemical sense to mean an energy generation process in which organic compounds act as both electron donors and terminal electron acceptors.

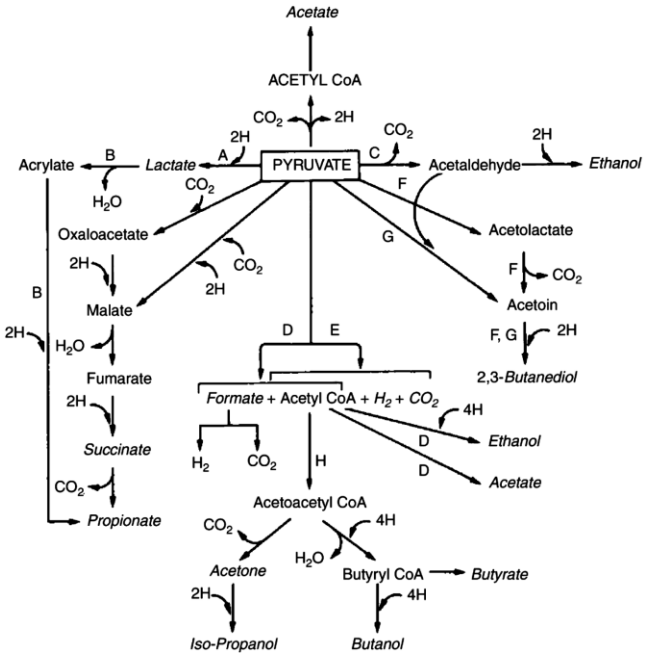

FIGURE 1.1 Bacterial Fermentation Products of Pyruvate

THE RANGE OF FERMENTATION PROCESSES

There are five major groups of commercially important fermentations:

1. Those that produce microbial cells (or biomass) as the product.

2. Those that produce microbial enzymes.

3. Those that produce microbial metabolites.

4. Those that produce recombinant products.

5. Those that modify a compound that is added to the fermentation—the transformation process.

MICROBIAL BIOMASS

The commercial production of microbial biomass may be divided into two major processes: the production of yeast to be used in the baking industry and the production of microbial cells to be used as human food or animal feed (single-cell protein). Bakers' yeast has been produced on a large scale since early 1900s and yeast was produced as human food in Germany during the First World War. However, it was not until the 1960s that the production of microbial biomass as a source of food protein was explored to any great depth. As a result of this work, a few large-scale continuous processes for animal feed production were established in the 1970s. These processes were based on hydrocarbon feedstocks, which could not compete against other high protein animal

feeds, resulting in their closure in the late 1980s. This process was based on a more stable economic platform and has been a significant economic success.

MICROBIAL ENZYMES

Enzymes have been produced commercially from plant, animal, and microbial sources. However, microbial enzymes have the enormous advantage of being able to be produced in large quantities by established fermentation techniques. Also, it is infinitely easier to improve the productivity of a microbial system compared with a plant or an animal one. Furthermore, the advent of recombinant DNA technology has enabled enzymes of animal origin to be synthesized by microorganisms. Enzyme production is closely controlled in microorganisms and in order to improve productivity these controls may have to be exploited or modified. Such control systems as induction may be exploited by including inducers in the medium, whereas repression control may be removed by mutation and recombination techniques. Also, the number of gene copies coding for the enzyme may be increased by recombinant DNA techniques.

MICROBIAL METABOLITES

The growth of a microbial culture can be divided into a number of stages. After the inoculation of a culture into a nutrient medium there is a period during which growth does not appear to occur; this period is referred as the lag phase and may be considered as a time of adaptation. Following a period during which the growth rate of the cells gradually increases, the cells grow at a constant maximum rate and this period is known as the log, or exponential, phase. Eventually, growth ceases and the cells enter the so-called stationary phase. After a further period of time, the viable cell number declines as the culture enters the death phase. As well as this kinetic description of growth, the behavior of a culture may also be described according to the products that it produces during the various stages of the growth curve. During the log phase of growth, the products produced are either anabolites (products of biosynthesis) essential to the growth of the organism and include amino acids, nucleotides, proteins, nucleic acids, lipids, carbohydrates, etc. or are catabolites (products of catabolism) such as ethanol and lactic acid, as illustrated in Fig. 1.1. These products are referred as the primary products of metabolism and the phase in which they are produced (equivalent to the log, or exponential phase) as the trophophase.

Many products of primary metabolism are of considerable economic importance and are being produced by fermentation. The synthesis of anabolic primary metabolites by wild-type microorganisms is such that their production is sufficient to meet the requirements of the organism. Thus, it is the task of the industrial microbiologist to modify the wild-type organism and to provide cultural conditions to improve the productivity of these compounds. This has been achieved very successfully, over many years, by the selection of induced mutants, the use of recombinant DNA technology, and the control of the process environment of the producing organism. This is exemplified by the production of amino acids where productivity has been increased by several orders of magnitude. However, despite these spectacular achievements, microbial processes have only been able to compete with the chemical industry for the production of relatively complex and high value compounds. In recent years, this situation has begun to change. The advances in metabolic engineering arising from genomics, proteomics, and metabolomics have provided new powerful techniques to further understand the physiology of "over-production" and to reengineer microorganisms to "over-produce" end products and intermediates of primary metabolism. Combined with the rising cost of petroleum and the desirability of environmentally friendly processes these advances are now facilitating the development of economic microbial processes for the production of bulk chemicals and feedstocks for the chemical industry.

During the deceleration and stationary phases, some microbial cultures synthesize compounds which are not produced during the trophophase and which do not appear to have any obvious function in cell metabolism. These compounds are referred to as the secondary compounds of metabolism and the phase in which they are produced (equivalent to the stationary phase) as the idiophase. It is important to realize that secondary metabolism may occur in continuous cultures at low growth rates and is a property of slow-growing, as well as nongrowing cells. When it is appreciated that microorganisms grow at relatively low growth rates in their natural environments, it is tempting to suggest that it is the idiophase state that prevails in nature rather than the trophophase, which may be more of a property of microorganisms in culture.

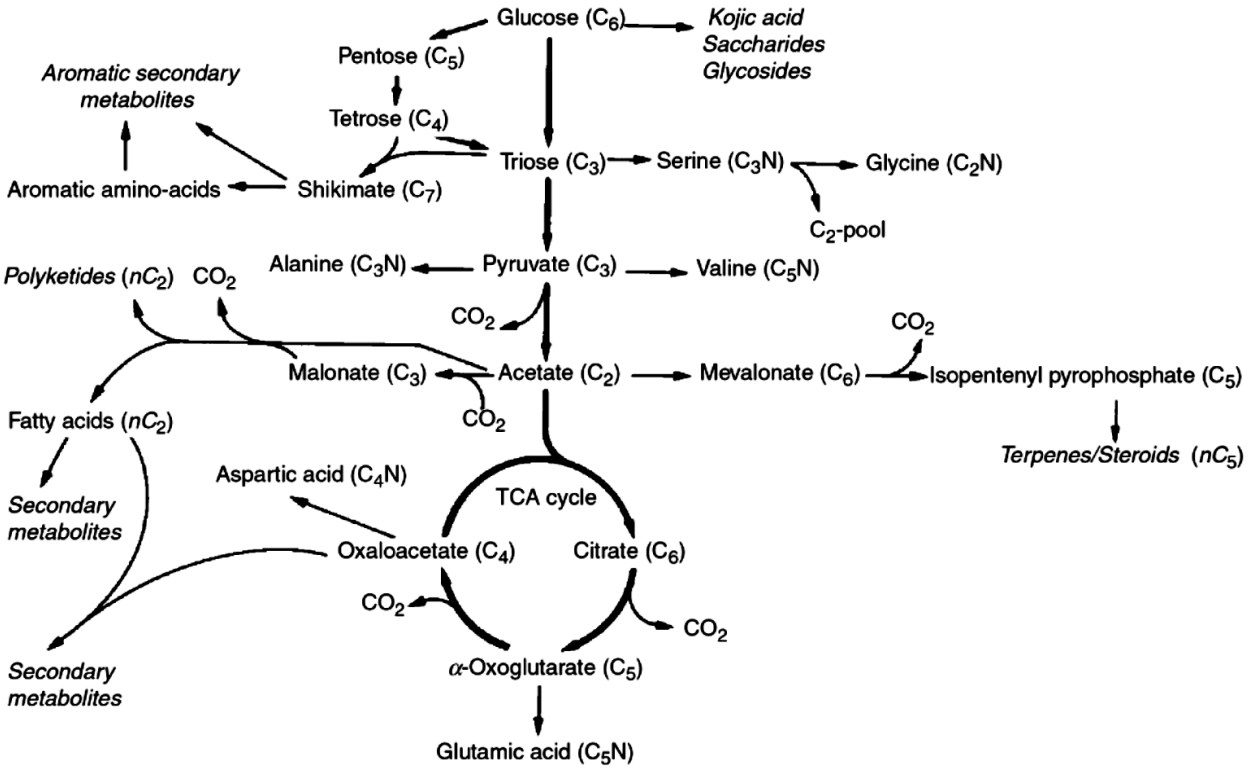

FIGURE 1.2 The Interrelationships Between Primary and Secondary Metabolism

The interrelationships between primary and secondary metabolism are illustrated in Fig. 1.2, from which it may be seen that secondary metabolites tend to be elaborated from the intermediates and products of primary metabolism. Although the primary biosynthetic routes illustrated in Fig. 1.2 are common to the vast majority of microorganisms, each secondary product would be synthesized by only a relatively few different microbial species. Thus, Fig. 1.2 is a representation of the secondary metabolism exhibited by a very wide range of different microorganisms. Also, not all microorganisms undergo secondary metabolism—it is common amongst microorganisms that differentiate such as the filamentous bacteria and fungi and the sporing bacteria but it is not found, for example, in the Enterobacteriaceae. Thus, the taxonomic distribution of secondary metabolism is quite different from that of primary metabolism. It is important to appreciate that the classification of microbial products into primary and secondary metabolites is a convenient, but in some cases, artificial system. The classification "should not be allowed to act as a conceptual straitjacket, forcing the reader to consider all products as either primary or secondary metabolites." It is sometimes difficult to categorize a product as primary or secondary and the kinetics of synthesis of certain compounds may change depending on the cultural conditions.

The physiological role of secondary metabolism in the producer organism in its natural environment has been the subject of considerable debate and their functions include effecting differentiation, inhibiting competitors, and modulating host physiology. However, the importance of these metabolites to the fermentation industry is the effects they have on organisms other than those that produce them. Many secondary metabolites have antimicrobial activity, others are specific enzyme inhibitors, some are growth promoters and many have pharmacological properties. Thus, the products of secondary metabolism have formed the basis of a major section of the fermentation industry. As in the case for primary metabolites, wild-type microorganisms tend to produce only low concentrations of secondary metabolites, their synthesis being controlled by induction, quorum sensing, growth rate, feedback systems, and catabolite repression, modulated by a range of effector molecules.

RECOMBINANT PRODUCTS

The advent of recombinant DNA technology has extended the range of potential fermentation products. Genes from higher organisms may be introduced into microbial cells such that the recipients are capable of synthesizing "foreign" proteins. These proteins are described as "heterologous" meaning "derived from a different organism." A wide range of microbial cells has been used as hosts for such systems including *Escherichia coli*, *Saccharomyces cerevisiae*, and filamentous fungi. Animal cells cultured in fermentation systems are also widely used for the production of heterologous proteins. Although the animal cell processes were based on microbial fermentation technology, a number of novel problems had to be solved—animal cells were considered extremely fragile compared with microbial cells, the achievable cell density is very much less than in a microbial process and the media are very complex.

Products produced by such genetically engineered organisms include interferon, insulin, human serum albumin, factors VIII and IX, epidermal growth factor, calf chymosin, and bovine somatostatin. Important factors in the design of these processes include the secretion of the product, minimization of the degradation of the product, and control of the onset of synthesis during the fermentation, as well as maximizing the expression of the foreign gene.

TRANSFORMATION PROCESSES

Microbial cells may be used to convert a compound into a structurally related, financially more valuable, compound. Because microorganisms can behave as chiral catalysts with high positional specificity and stereospecificity, microbial processes are more specific than purely chemical ones and enable the addition, removal, or modification of functional groups at specific sites on a complex molecule without the use of chemical protection. The reactions, which may be catalyzed include dehydrogenation, oxidation, hydroxylation, dehydration and condensation, decarboxylation, animation, deamination, and isomerization. Microbial processes have the additional advantage over chemical reagents of operating at relatively low temperatures and pressures without the requirement for potentially polluting heavy-metal catalysts. Although the production of vinegar is the oldest established microbial transformation process (conversion of ethanol to acetic acid), the majority of these processes involve the production of high-value compounds including steroids, antibiotics, and prostaglandins.

However, the conversion of acetonitrile to acrylamide by *Rhodococcus rhodochrous* is an example of the technology being used in the manufacturing of a bulk chemical—20,000 metric tons being produced annually. A novel application of microbial transformation is the use of microorganisms to mimic mammalian metabolism. Humans and animals will metabolize drugs such that they may be removed from the body. The resulting metabolites may be biologically active themselves—either eliciting a desirable effect or causing damage to the organism. Thus, in the development of a drug it is necessary to determine the activity of not only the administered drug but also its metabolites. These studies may require significant amount of the metabolites and while it may be possible to isolate them from tissues, blood, urine, or faeces of the experimental animal, their concentration is often very low resulting in such approaches being time-consuming, expensive, and far from pleasant. The exploitation of the metabolic ability of microorganisms to perform these biotransformations. Thus, drug metabolites have been produced in small-scale fermentation, facilitating the investigation of their biological activity and/or toxicity. The anomaly of the transformation fermentation process is that a large biomass has to be produced to catalyze a single reaction. Thus, many processes have been streamlined by immobilizing either the whole cells, or the isolated enzymes, which catalyze the reactions, on an inert support. The immobilized cells or enzymes may then be considered as catalysts, which may be reused many times.

THE CHRONOLOGICAL DEVELOPMENT OF THE FERMENTATION INDUSTRY

The chronological development of the fermentation industry may be represented as five overlapping stages. The development of the industry prior to 1900 is represented by stage 1, where the products were confined to potable alcohol and vinegar. Although beer was first brewed by the ancient Egyptians, the first true large-scale breweries date from the early 1700s when wooden vats of 1500 barrels capacity were introduced. Even some process control was attempted in these early breweries, as indicated by the recorded use of thermometers in 1757 and the development of primitive heat exchangers in 1801. By the mid-1800s, the role of yeasts in alcoholic fermentation had been demonstrated independently by Cagniard-Latour, Schwann, and Kutzing but it was Pasteur who eventually convinced the scientific world of the obligatory role of these microorganisms in the process. During the late 1800s, Hansen started his pioneering work at the Carlsberg brewery and developed methods for isolating and propagating single yeast cells to produce pure cultures and established sophisticated techniques for the production of starter cultures. However, use of pure cultures did not spread to the British ale breweries and it is true to say that many of the small, traditional, ale-producing breweries still use mixed yeast cultures at the present time but, nevertheless, succeed in producing high quality products.

Table 1.1 The Stages in the Chronological Development of the Fermentation Industry

Stage	Main Products	Vessels	Process Control	Culture Method	Quality Control	Pilot Plant Facilities	Strain Selection
1 Pre-1900	Alcohol	Wooden, up to 1500 barrels capacity Copper used in later breweries	Use of thermometer, hydrometer and heat exchangers	Batch	Virtually nil	Nil	Pure yeast cultures used at the Carlsberg brewery (1886)
	Vinegar	Barrels, shallow trays, trickle filters		Batch	Virtually nil	Nil	Fermentations inoculated with 'good' vinegar
2 1900–1940	Bakers' yeast glycerol, citric acid, lactic acid and acetone/butanol	Steel vessels of up to 200 m^3 for acetone/butanol Air spargers used for bakers' yeast Mechanical stirring used in small vessels	pH electrodes with off-line control Temperature control	Batch and fed-batch systems	Virtually nil	Virtually nil	Pure cultures used
3 1940–date	Penicillin, streptomycin, other antibiotics, gibberellin, amino acids, nucleotides, transformations, enzymes	Mechanically aerated vessels, operated aseptically—true fermenters	Sterilizable pH and oxygen electrodes. Use of control loops which were later computerized	Batch and fed-batch common Continuous culture introduced for brewing and some primary metabolites	Very important	Becomes common	Mutation and selection programmes essential
4 1964–date	Single-cell protein using hydrocarbon and other feedstocks	Pressure cycle and pressure jet vessels developed to overcome gas and heat exchange problems	Use of computer linked control loops	Continuous culture with medium recycle	Very important	Very important	Genetic engineering of producer strains attempted
5 1982–date	Production of heterologous proteins by microbial and animal cells Monoclonal antibodies produced by animal cells	Fermenters developed in stages 3 and 4. Animal cell reactors developed	Control and sensors developed in stages 3 and 4	Batch, fed-batch or continuous Continuous perfusion developed for animal cell processes	Very important	Very important	Introduction of foreign genes into microbial and animal cell hosts. In vitro recombinant DNA techniques used in the improvement of stage 3 products
6 2000–date	Use of "synthetic biology" to improve established fermentations and develop new bulk chemical processes	Fermenters developed in stages 3 and 4	Control and sensors developed in stages 3 and 4	Batch, fed-batch or continuous	Very important	Very important	Synthetic biology used to develop existing and novel fermentations

THE COMPONENT PARTS OF A FERMENTATION PROCESS

Regardless of the type of fermentation (with the possible exception of some transformation processes) an established process may be divided into six basic component parts:

1. The formulation of media to be used in culturing the process organism during the development of the inoculum and in the production fermenter.

2. The sterilization of the medium, fermenters, and ancillary equipment.

3. The production of an active, pure culture in sufficient quantity to inoculate the production vessel.

4. The growth of the organism in the production fermenter under optimum conditions for product formation.

5. The extraction of the product and its purification.

6. The disposal of effluents produced by the process. The interrelationships between the six component parts are illustrated in Fig. 1.3. However, one must also visualize the research and development program which is designed to gradually improve the overall efficiency of the fermentation. Before a fermentation process is established a producer organism has to be isolated, modified such that it produces the desired product in commercial quantities, its cultural requirements determined and the plant designed accordingly. Also, the extraction process has to be established. The development program would involve the continual improvement of the process organism, the culture medium, and the extraction process.

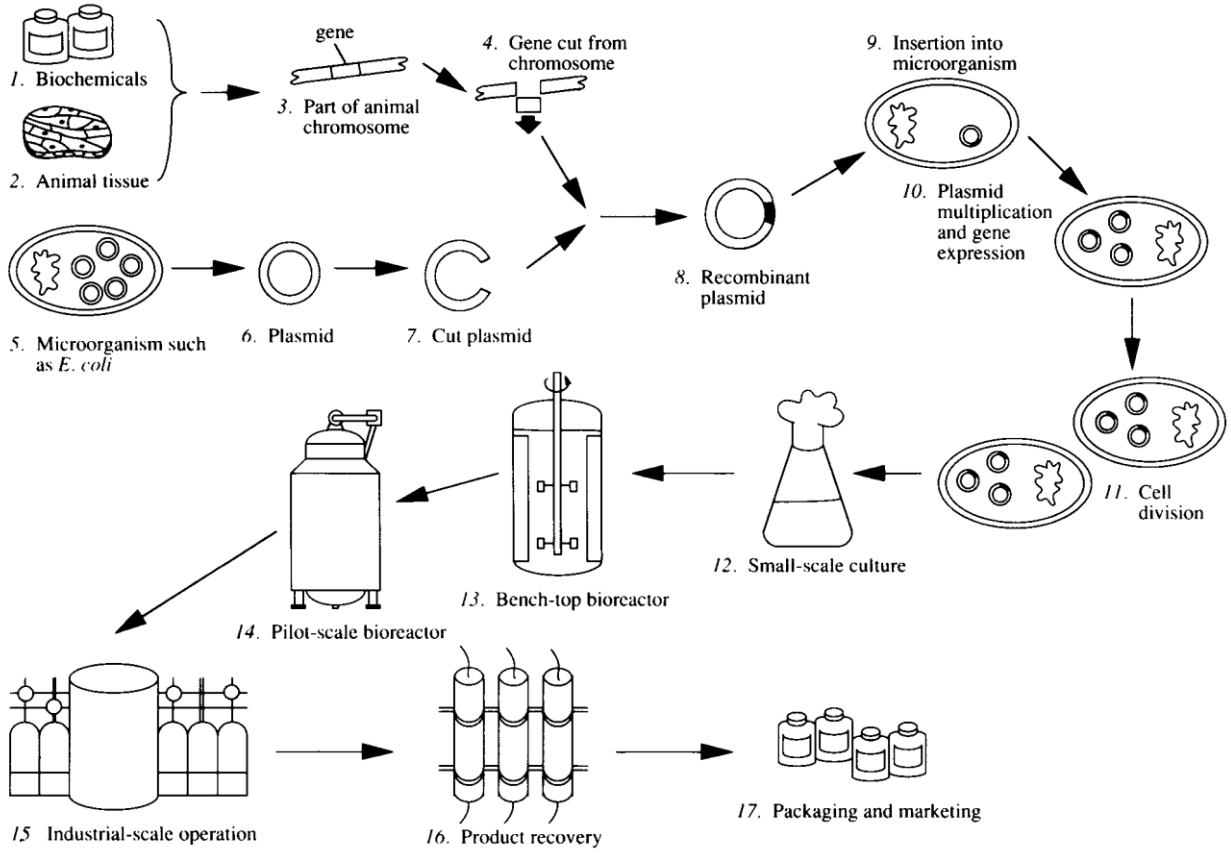

FIGURE 1.3 A Generalized Schematic Representation of a Typical Fermentation Process

ISOLATION OF INDUSTRIALLY IMPORTANT MICROORGANISMS

The remarkable biochemical diversity of microorganisms has been exploited by the fermentation industry by isolating strains from the natural environment able to produce products of commercial value. The so-called “golden era” of antibiotic discovery in the 1950s and 1960s, culturing soil microorganisms and screening their products, yielded the key groups of antibiotics currently in use.

Unfortunately, the application of combinatorial chemistry was not the answer to this prayer and the industry gained relatively little from enormous effort and expenditure. The details of this scenario will be considered in a later section as well as the contribution that microbial genomics made to the next stage of natural product exploration. However, the “golden age” gave rise to much of the industry and it should be remembered that antibiotics are not the only natural products produced by microorganism isolated from soil. Thus, the basic principles of strain isolation will be considered to give an appreciation of the origin of many current industrial producers and the basis of future developments.

The first stage in the screening for microorganisms of potential industrial application is their isolation. Isolation involves obtaining either pure or mixed cultures followed by their assessment to determine which carry out the desired reaction or produce the desired product. In some cases it is possible to design the isolation procedure in such a way that the growth of potential producers is encouraged or that they may be recognized at the isolation stage, whereas in other cases organisms must be isolated and producers recognized at a subsequent stage. However, it should be remembered that the isolate must eventually carry out the process economically and therefore the selection of the culture to be used is a compromise between the productivity of the organism and the economic constraints of the process. Bull, Ellwood, and Ratledge (1979) cited a number of criteria as being important in the choice of organism:

1. The nutritional characteristics of the organism. Depending on the value of the product, a process may have to be carried out using a cheap medium or a predetermined one, for example, the use of methanol as an energy source. These requirements may be met by the suitable design of the isolation medium.
2. The optimum temperature of the organism. The use of an organism having an optimum temperature above 40°C considerably reduces the cooling costs of a large-scale

fermentation and, therefore, the use of such a temperature in the isolation procedure may be beneficial.

3. The reaction of the organism with the equipment to be employed and the suitability of the organism to the type of process to be used.
4. The stability of the organism and its amenability to genetic manipulation.
5. The productivity of the organism, measured in its ability to convert substrate into product and to give a high yield of product per unit time.
6. The ease of product recovery from the culture.

Points 3, 4, and 6 would have to be assessed in detailed tests subsequent to isolation and the organism most well suited to an economic process chosen on the basis of these results. However, before the process may be put into commercial operation the toxicity of the product and the organism has to be assessed. The aforementioned account implies that cultures must be isolated, in some way, from natural environments. However, the industrial microbiologist may also "isolate" microorganisms from culture collections. A selection of the major collections is given in Table 3.1. Such collections may provide organisms of known characteristics but may not contain those possessing the most desirable features, whereas the environment contains a myriad of organisms, very few of which may be satisfactory. It is certainly cheaper to buy a culture than to isolate from nature, but it is also true that a superior organism may be found after an exhaustive search of a range of natural environments. However, it is always worthwhile to purchase cultures demonstrating the desired characteristics, however weakly, as they may be used as model systems to develop culture and assay techniques that may then be applied to the assessment of natural isolates.

The ideal isolation procedure commences with an environmental source (frequently soil) which is highly probable to be rich in the desired types, is so designed as to favor the growth of those organisms possessing the industrially important characteristic (ie, the industrially useful characteristic is used as a selective factor) and incorporates a simple test to distinguish the most desirable types. Selective pressure may be used in the isolation of organisms that will grow on particular substrates, in the presence of certain compounds or under cultural conditions adverse to other types. However, if it is not possible to apply selective pressure for the desired character, it

may be possible to design a procedure to select for a microbial taxon which is known to show the characteristic at a relatively high frequency, for example, the production of antibiotics by streptomycetes. Alternatively, the isolation procedure may be designed to exclude certain microbial "weeds" and to encourage the growth of more novel types. Indeed, as pointed out by Bull, Goodfellow, and Slater (1992), for screening programs to continue to generate new products, it is becoming increasingly more important to concentrate on lesser known microbial taxa or to utilize very specific screening tests to identify the desired activity. During the 1980s significant advances were made in the establishment of taxonomic databases describing the properties of microbial groups and these databases have been used to predict the cultural conditions that would select for the growth of particular taxa. Thus, the advances in the taxonomic description of taxa have allowed the rational design of procedures for the isolation of strains that may have a high probability of being productive or are representatives of unusual groups. Furthermore, wholegenome sequencing of microorganisms (developed in the 2000s) can give an insight into their cultural requirements (de Macedo Lemos, Alves, & Campanharo, 2003). The sequence can be used to construct a metabolic map and thus identify nutrients that the organism cannot synthesize (Song et al., 2008), thereby enabling the design of a defined medium. To assist this approach, Richards et al. (2014) constructed a database (MediaDB) of defined media that have been used for the cultivation of organisms with sequenced genomes. The advances in pharmacology and molecular biology have also enabled the design of screening tests to identify productive strains among the isolated organisms. However, as will be discussed later, this approach did not live up to expectations in the identification of novel antibiotics.

ISOLATION METHODS UTILIZING SELECTION OF THE DESIRED CHARACTERISTIC

In this section we consider methods that take direct advantage of the industrially relevant property exhibited by an organism to isolate that organism from an environmental source. Isolation methods depending on the use of desirable characteristics as selective factors are essentially types of enrichment culture. Enrichment culture is a technique resulting in an increase in the number of a given organism relative to the numbers of other types in the original inoculum. The process involves taking an environmental source (usually soil) containing a mixed population and providing conditions either suitable for the growth of the desired type, or unsuitable for the growth of the other organisms, for example, by the provision of particular substrates or the inclusion of

certain inhibitors. Prior to the culture stage it is often advantageous to subject the soil to conditions that favor the survival of the organisms in question. For example, air-drying the soil will favor the survival of actinomycetes.

Enrichment liquid culture

Enrichment liquid culture is frequently carried out in shake flasks. However, the growth of the desired type from a mixed inoculum will result in the modification of the medium and therefore changes the selective force which may allow the growth of other organisms, still viable from the initial inoculum, resulting in a succession. The selective force may be reestablished by inoculating the enriched culture into identical fresh medium. Such subculturing may be repeated several times before the dominant organism is isolated by spreading a small inoculum of the enriched culture onto solidified medium. The time of subculture in an enrichment process is critical and should correspond to the point at which the desired organism is dominant. The prevalence of an organism in a batch enrichment culture will depend on its maximum specific growth rate compared with the maximum specific growth rates of the other organisms capable of growth in the inoculum. Thus, provided that the enrichment broth is subcultured at the correct times, the dominant organism will be the fastest growing of those capable of growth. However, it is not necessarily true that the organism with the highest specific growth rate is the most useful, for it may be desirable to isolate the organism with the highest affinity for the limiting substrate.

The problems of time of transfer and selection on the basis of maximum specific growth rate may be overcome by the use of a continuous process where fresh medium is added to the culture at a constant rate. Under such conditions, the selective force is maintained at a constant level and the dominant organism will be selected on the basis of its affinity for the limiting substrate rather than its maximum growth rate. The basic principles of continuous culture are considered in Chapter 2 from which it may be seen that the growth rate in continuous culture is controlled by the dilution rate and is related to the limiting substrate concentration by the equation:

$$\mu = \mu\, s/(K + s).\ \max\ s \quad (1.1)$$

Eq. (1.1) is represented graphically in Fig. 1.1. A model of the competition between two organisms capable of growth in a continuous enrichment culture is represented in Fig. 1.2. Consider the behavior of the two organisms, A and B, in Fig. 1.2. In continuous culture the specific growth rate

is determined by the substrate concentration and is equal to the dilution rate, so that at dilution rates below point Y in Fig. 1.2 strain B would be able to maintain a higher growth rate than strain A, whereas at dilution rates above Y strain A would be able to maintain a higher growth rate. Thus, if A and B were present in a continuous enrichment culture, limited by the substrate depicted in Fig. 1.2, strain A would be selected at dilution rates above

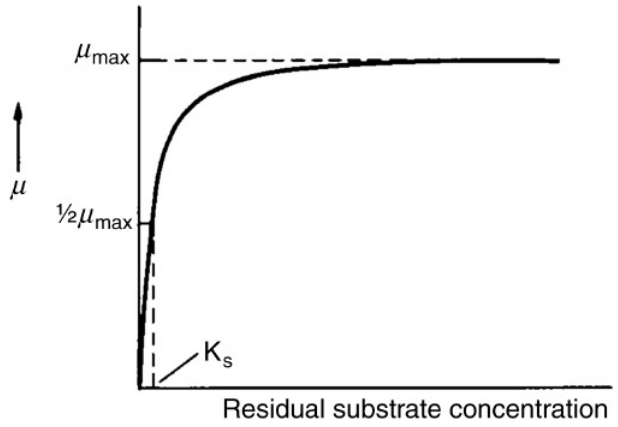

FIGURE 1.1 The Effect of Substrate Concentration on the Specific Growth Rate of a Microorganism

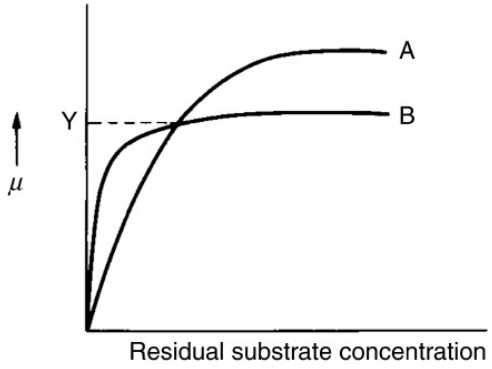

FIGURE 1.2 The Effect of Substrate Concentration on the Specific Growth Rates of Two Microorganisms A and B

Y and strain B would be selected at dilution rates below Y. Thus, the organisms that are isolated by continuous enrichment culture will depend on the dilution rate employed and may result in the isolation of organisms not so readily recovered by batch techniques. Continuous enrichment techniques are especially valuable in isolating organisms to be used in a continuous-flow commercial process. Organisms isolated by batch enrichment and purification on solid media frequently perform poorly in continuous culture (Harrison, Wilkinson, Wren, & Harwood, 1976), whereas continuous enrichment provides an organism, or mixture of organisms, adapted to

continuous culture. The enrichment procedure should be designed such that the predicted isolate meets as many of the criteria of the proposed process as possible and both Johnson (1972) and Harrison (1978) have discussed such procedures for the isolation of organisms to be used for biomass production. Johnson emphasized the importance of using the carbon source to be employed in the subsequent commercial process as the sole source of organic carbon in the enrichment medium, and that the medium should be carbon limited. The inclusion of other organic carbon sources, such as vitamins or yeast extract, may result in the isolation of strains adapted to using these, rather than the principal carbon source, as energy sources. The isolation of an organism capable of growth on a simple medium should also form the basis of a cheaper commercial process and should be more resistant to contamination—a major consideration in the design of a commercial continuous process. The use of as high as possible an isolation temperature should also result in the isolation of a strain presenting minimal cooling problems in the subsequent process.

The main difficulty in using a continuous-enrichment process is the washout of the inoculum before an adapted culture is established. Johnson (1972) suggested that the isolation process should be started in batch culture using a 20% inoculum and as soon as growth is observed, the culture should be transferred to fresh medium and the subsequent purification and stabilization of the enrichment performed in continuous culture. The continuous system should be periodically inoculated with soil or sewage that may not only be a source of potential isolates but should also ensure that the dominant flora is extremely resistant to contamination.

Harrison (1978) proposed two solutions to the problem of early washout in continuous isolation processes: The first uses a turbidostat and the second uses a twostage chemostat. A turbidostat is a continuous-flow system provided with a photoelectric cell to determine the turbidity of the culture and maintain the turbidity between set points by initiating or terminating the addition of medium. Thus, washout is avoided, as the medium supply will be switched off if the biomass falls below the lower fixed point. The use of a turbidostat will result in selection on the basis of maximum specific growth rate as it operates at high levels of limiting substrate. Thus, although the use of the turbidostat removes the danger of washout it is not as flexible a system as the chemostat that may be used at a range of dilution rates. The two-stage chemostat described and the first stage of the system was used as a continuous inoculum for the second stage and consisted of a large bottle containing a basic medium inoculated with a soil infusion. Continuous inoculation

was employed until an increasing absorbance was observed in the second stage. The use of feedback continuous systems for the isolation of strains with particularly high affinity for substrate and this approach would also guard against premature washout. The use of continuous enrichment culture has frequently resulted in the selection of stable, mixed cultures presumably based on some form of symbiotic relationship. It is extremely unlikely that such mixed, stable systems could be isolated by batch techniques so that the adoption of continuous enrichment may result in the development of novel, mixed culture fermentations. Harrison, Topiwala, and Hamer (1972) isolated a mixed culture using methane as the carbon source in a continuous enrichment and demonstrated that the mixture contained one methylotroph and a number of nonmethylotrophic symbionts. The performance of the methylotroph in pure culture was invariably poorer than the mixture in terms of growth rate, yield, and culture stability.

Continuous enrichment has also been used for the isolation of organisms to be used in systems other than biomass production; Rowley and Bull (1977) used the technique to isolate an *Arthrobacter* sp. producing a yeast lysing enzyme complex. The technique has been used widely for the isolation of strains capable of degrading environmental pollutants. For example, Futamata, Nagano, Watanabe, and Hiraishi (2005) isolated a number of *Variovax* strains able to degrade trichloroethylene.

Enrichment cultures using solidified media

Solidified media have been used for the isolation of certain enzyme producers and these techniques usually involve the use of a selective medium incorporating the substrate of the enzyme that encourages the growth of the producing types. Aunstrup, Outtrup, Andresen, and Dambmann (1972) isolated species of *Bacillus* producing alkaline proteases. Soils of various pHs were used as the initial inoculum and, to a certain extent, the number of producers isolated correlated with the alkalinity of the soil sample. The soil samples were pasteurized to eliminate nonsporulating organisms and then spread onto the surface of agar media at pH 9–10, containing a dispersion of an insoluble protein. Colonies that produced a clear zone due to the digestion of the insoluble protein were taken to be alkaline protease producers. The size of the clearing zone could not be used quantitatively to select high producers, as there was not an absolute correlation between the size of the clearing zone and the production of alkaline protease in submerged culture. However,

this example demonstrates the importance of choice of starting material, the use of a selective force in the isolation and the incorporation of a preliminary diagnostic test, albeit of limited use.

The Preservation of Industrially Important Microbes

Introduction

Microbes are required for the production of fermentation products. They are very valuable for specific product. Not all the microbes will give one product produced efficiently by specific microbe.

The isolation of a desired organism for a fermentation process may be time consuming and very expensive procedure and it is therefore essential that it retain the desirable characteristics that led to its selection. In addition, the culture used for the fermentation process should remain viable and free from contamination. Thus, industrial cultures must be preserved and maintained in such way as to eliminate genetic change, protect against contamination, and retain viability.

Different techniques are used for maintenance and preservation of different organisms based on their properties. Selected method should also conserve the properties of the organisms.

Techniques for the Preservation of microbes broadly divided into two

1. Methods where organisms are in Continuous metabolic active state
2. Methods where organisms are in Suspended metabolic state

1. Continuous metabolic active state preservation technique

In this technique, organisms preserved on nutrient medium by repeated sub-culturing. In this technique, any organisms are stored by using general nutrient medium. Here repeated sub-culturing is required due to depletion or drying of nutrient medium. This technique includes preservation by following methods.

1.1 Periodic transfer to fresh media

Organisms grown in general media on slant, incubated for particular period at particular temperature depending on the characteristics of the selected organisms, then it is stored in refrigerator. These cultures can be stored for certain interval of time depending on the organism and its growth conditions. After that time interval, again these organisms transferred to new fresh medium and stored in refrigerator.

1.2 Overlaying culture with mineral oil

Organisms are grown on agar slant then they are covered with sterile mineral oil to a depth of 1 cm. above the tip of the surface. This method is simple; one can remove some organisms in aseptic

condition with the help of sterile wire loop and still preserving the initial culture. Some species preserved satisfactorily for 15 – 20 years by this method.

1.3 Storage in sterile soil

This method is widely used for preserving spore forming bacteria and fungi. In this method, organisms will remain in dormant stage in sterile soil. Soil sterilized then spore suspension added to it aseptically, this mixture dried at room temperature and stored in refrigerator. Viability of organisms found around 70 – 80 years.

1.4 Saline suspension

Normal Saline used to provide proper osmotic pressure to organism's otherwise high salt concentration is inhibitory for organisms. Organisms kept in screw cap bottles in normal saline, stored at room temperature, wherever required transfer made on agar slats, and incubated.

2 Methods where organisms are in Suspended metabolic state

Organisms preserved in suspended metabolic state by either drying or storing at low temperature. Microbes are dried or kept at low temperature carefully so that their revival is possible.

2.1 Drying in vacuum

In this technique, organisms dried over chemical instead of air dry. Cells passed over CaCl2 in a vacuum and then stored in refrigerator. Organisms survive for longer period.

2.2 Lyophilization

Lyophilization is vacuum sublimation technique. Cells grown in nutritive media and then this culture distributed in small vials. These vials culture then immersed in a mixture of dry ice and alcohol at -78oC. These vials immediately connected to a high-vacuum line, and when they are completely dried, each vial sealed under vacuum. This is most effective and widely

Industrial enzymes are enzymes that are commercially used in a variety of industries such as pharmaceuticals, chemical production, biofuels, food & beverage, and consumer products. Due to advancements in recent years, biocatalysis through isolated enzymes is considered more economical than use of whole cells. Enzymes may be used as a unit operation within a process to generate a desired product, or may be the product of interest. Industrial biological catalysis through enzymes has experienced rapid growth in recent years due to their ability to operate at mild conditions, and exceptional chiral and positional specificity, things that traditional chemical processes lack. Isolated enzymes are typically used in hydrolytic and isomerization reactions. Whole cells are typically used when a reaction requires a co-factor. Although co-factors may be generated in vitro, it is typically more cost-effective to use metabolically active cells.

Enzymes as a unit operation

Immobilization

Despite their excellent catalytic capabilities, enzymes and their properties must be improved prior to industrial implementation in many cases. Some aspects of enzymes that must be improved prior to implementation are stability, activity, inhibition by reaction products, and selectivity towards non-natural substrates. This may be accomplished through immobilization of enzymes on a solid material, such as a porous support. Immobilization of enzymes greatly simplifies the recovery process, enhances process control, and reduces operational costs. Many immobilization techniques exist, such as adsorption, covalent binding, affinity, and entrapment. Ideal immobilization processes should not use highly toxic reagents in the immobilization technique to ensure stability of the enzymes. After immobilization is complete, the enzymes are introduced into a reaction vessel for biocatalysis.

Adsorption

Enzyme adsorption onto carriers functions based on chemical and physical phenomena such as van der Waals forces, ionic interactions, and hydrogen bonding. These forces are weak, and as a result, do not affect the structure of the enzyme. A wide variety of enzyme carriers may be used. Selection of a carrier is dependent upon the surface area, particle size, pore structure, and type of functional group.

Covalent binding

Example of Enzyme Immobilization through Covalent Binding

Many binding chemistries may be used to adhere an enzyme to a surface to varying degrees of success. The most successful covalent binding techniques include binding via glutaraldehyde to amino groups and N-hydroxysuccinide esters. These immobilization techniques occur at ambient temperatures in mild conditions, which have limited potential to modify the structure and function of the enzyme.

Affinity

Immobilization using affinity relies on the specificity of an enzyme to couple an affinity ligand to an enzyme to form a covalently bound enzyme-ligand complex. The complex is introduced into a support matrix for which the ligand has high binding affinity, and the enzyme is immobilized through ligand-support interactions.

Entrapment

Immobilization using entrapment relies on trapping enzymes within gels or fibers, using non-covalent interactions. Characteristics that define a successful entrapping material include high surface area, uniform pore distribution, tunable pore size, and high adsorption capacity.

Batch processes

In a batch process, all nutrients are provided at the beginning of the cultivation, without adding any more in the subsequent bioprocess. During the entire bioprocess, no additional nutrients are added – just control elements such as gases, acids and bases; it is a closed system. The bioprocess then lasts until the nutrients are consumed. This strategy is suitable for rapid experiments such as strain characterization or the optimization of nutrient medium. The disadvantage of this convenient method is that the biomass and product yields are limited. Since the carbon source and/or oxygen transfer are usually the limiting factor, the microorganisms are not in the exponential growth phase for a long time.

To improve the availability of dissolved oxygen, the oxygen transfer rate must be increased. This is achieved by increasing the stirring speed, the gas flow, the proportion of oxygen in the gas mix, or the pressure (if the bioprocess takes place in a steel bioreactor). Since the combination of the various parameters is intended to improve the concentration, sophisticated management and control processes of these parameters are needed. These processes, known as cascades, can be configured individually to adapt them to a specific application. One or more parameters that are

supposed to be used for adjusting the concentration of dissolved oxygen are predefined at the controller. The first step towards reaching the target value is to vary the first parameter (for example stirrer speed) within the defined range. If that does not work, the next step is to change subsequent parameters until the target value can be maintained.

After the end of a bioprocess run in batch mode, only the biomass or medium is harvested and appropriately processed to obtain the desired product. From the bioreactor point of view, the process is repeatedly interrupted by cleaning and sterilization steps, and the biomass is only produced in stages. In addition to the low yield of biomass, batch processes have also an increased risk for substrate or product inhibition. The latter describes the interference of enzyme activity by the presence of high concentrations of substrate or product, which might induce metabolic feedback that can drastically reduce the yield.

The advantages of a batch culture are:

Short duration

Less chance of contamination as no nutrients are added

Separation of batch material for traceability

Easier to manage

Some disadvantages include:

Product is mixed in with nutrients, reagents, cell debris and toxins

Shorter productive time

Can involve storage of batches for downstream processing

Fed-batch processes

One way of keeping nutrients from becoming a limiting factor is to constantly supply them during cultivation. This is called a fed-batch process, which is a partly open system. The advantage of feeding during cultivation is that it allows to overall achieve higher product quantities overall. Under specific growth conditions, the microorganisms and/or cells constantly double and therefore follow an exponential growth curve. This is why the feed rate should increase exponentially as well. Generally, the substrate is pumped from the supply bottle into the culture vessel through a silicone tube. The user can either manually set the feed at any time (linear, exponential, pulse-

wise), or add nutrients when specific conditions are met, such as when a certain biomass concentration is reached or when a nutrient is depleted.

The fed-batch process offers a wide range of control strategies and is also suitable for highly specialized applications. However, it may increase the processing time and potentially leads to inhibition through the accumulation of toxic by-products. The user also needs to have a more in-depth understanding of bioprocesses to do this, which should, however, not be interpreted as a disadvantage.

While the batch process is classified as a discontinuous process, a fed-batch process is a semi-continuous process. During experiments at the beginning of the last century with the aim to produce as much biomass as possible from baker's yeast in a batch process, excessively high substrate concentrations (in this case glucose) were found to inhibit growth, mainly by the formation of ethanol. On the other hand, this property of baker's yeast can be used to produce ethanol. At high glucose concentrations and sufficient dissolved oxygen in the medium, alcoholic fermentation still occurs, which is called the Crabtree effect. This effect is used in some food production processes with yeast.

Due to their advantages, fed-batch processes per se are now used in all areas of biotechnological production, in particular for the production of recombinant proteins and antibiotics.

The advantages of a fed-batch culture are:

Extends a culture's productive duration

Can be used to switch genes on or off by changing substrate

Can be manipulated for maximum productivity using different feeding strategies

Some disadvantages include:

Allows build up of inhibitory agents and toxins

Provides another point of ingress for contamination

May produce high cell density numbers and product yields which are difficult to deal within downstream, creating bottlenecks in the whole process.

Continuous culture

After a batch growth phase, an equilibrium is established with respect to a particular component (also called steady state). Under these conditions, as much fresh culture medium is added, as it is

removed (chemostat). These bioprocesses are referred to as continuous cultures, and are particularly suitable when an excess of nutrients would result in inhibition due to e.g. toxin build up or excessive heating. Other advantages of this method include reduced product inhibition and an improved space-time yield. When medium is removed, cells are harvested, which is why the inflow and outflow rates must be less than the doubling time of the microorganisms. Alternatively, the cells can be retained in a wide variety of ways (for example, in a spin filter), which is called perfusion. In a continuous process, the space-time yield of the bioreactor can be even further improved compared to that of a fed-batch process. However, the long cultivation period also increases the risk of contamination and long-term changes in the cultures. Moreover, continuous processes are ideal tools for gaining a better understanding of the process, since all process parameters remain constant when the system is operating correctly.

The three most common types of continuous culture are:

Chemostat: The rate of addition of a single growth-limiting substrate controls cell multiplication.

Turbidostat: An indirect measurement of cell numbers (turbidity or optical density) controls addition and removal of liquid. This needs an additional sensor but is driven by real-time feedback.

Perfusion: This type of continuous bioprocessing mode is based on either retaining the cells in the bioreactor or recycling the cells back to the bioreactor. Fresh medium is provided and cell-free supernatant gets removed at the same rate.

The advantages of a continuous culture are:

Allows the maximum productivity

Time for cleaning, sterilisation and handling of the vessel are all reduced

Provides a steady state for metabolic studies when many elements sum to zero

Some disadvanteages include:

Difficult to keep a constant population density over prolonged periods

The products of a continuous process cannot be neatly separated into batches for traceability

Increased risk of contamination and genetic changes

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UNIT – II– Bioprocess Engineering -1 – SBT1301

Medium requirement for fermentation processes

Introduction

Fermentation technology is widely used for the production of various economically important compounds which have applications in the energy production, pharmaceutical, chemical and food industry. Although, fermentation processes are used from generations, the need for sustainable production of products, meet the market requirements in a cost effective manner has put forward a challenging demand. For any fermentation based product, the most important thing is the availability of fermented product equal to that of market demand. Various microorganisms have been reported to produce an array of primary and secondary metabolites, but in a very low quantity. In order to meet the market demand, several high yielding techniques have been discovered in the past, and successfully implemented in various processes, like production of primary or secondary metabolites, biotransformation, oil extraction etc.

Medium optimization is still one of the most critically investigated phenomenon that is carried out before any large scale metabolite production, and possess many challenges too. Before 1970s, media optimization was carried out by using classical methods, which were expensive, time consuming, involving plenty of experiments with compromised accuracy. Nevertheless, with the advent of modern mathematical/statistical techniques, media optimization has become more vibrant, effective, efficient, economical and robust in giving the results. For designing a production medium, the most suitable fermentation conditions (e.g., pH, temperature, agitation speed, etc.) and the appropriate medium components (e.g., carbon, nitrogen, etc.) must be identified and optimized accordingly. Further, by optimizing the above said parameters, maximum product concentration could be achieved. The schematic representation of a systematic approach of fermentation medium designing has been given in Figure 1.

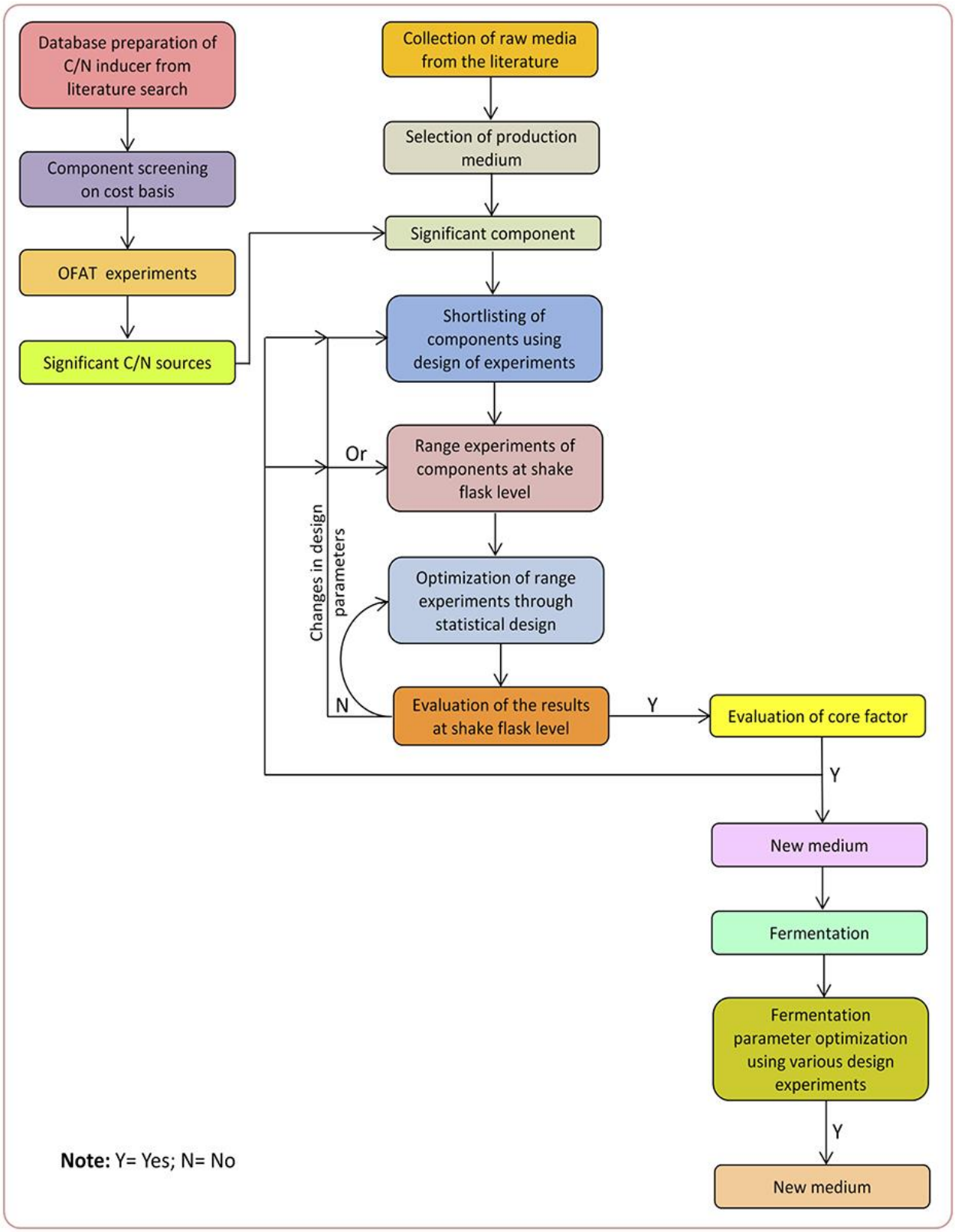


Figure 1. Schematic diagram of a systematic approach of fermentation medium designing.

An increase in productivity reduces the overall cost of the product, as well as the production cost; hence, it is one of the important topics for the research. Usually, enhanced productivity can be achieved either by strain improvement or by optimizing the process parameters. But, strain improvement and optimization are “Catch-22” situation. You cannot chose a lead strain until you have the best medium and you cannot propose a finest medium until you have the lead strain. Usually, the researchers around the world solve this predicament by sticking to one component at a time. However, both strategies cannot guarantee that one of the preferred strain if another medium is used. With this drawback and Catch-22 situation, various new methods have been suggested and investigated, where both the medium design and strain improvement can be carried out simultaneously.

In this review we have restricted our scope and discussed about the media formulation and media optimization techniques in terms of their utility, application and feasibility to maximize the metabolite yield produced by the fermentation process. In order to provide clarity and better understanding for the readers, initially we have discussed the roles of various (major) components of the fermentation media, followed by detailed description of statistical/mathematical optimization techniques. Also, the advantages and disadvantages associated with the above methods along with the future directions in the fermentation media design and optimization have been discussed in detail.

Nutritional Control of Metabolite Production

Fermented products that are used in our daily life are either primary or secondary metabolites produced during the trophophase and idiophase of the microbial growth, respectively. High productivity titer is the pre-requisite for the industrial production of any type of metabolite. The production of specific metabolites in high titer could be possible by maintaining proper control and regulation at different levels via transport and metabolism of extra-cellular nutrients, precursor formation and accumulation of intermediates. Fermentation processes, where the precursor(s) of the specific products are not added in the medium, carbon and nitrogen sources present in the medium during their metabolism may initiate the biosynthesis of precursors that regulate the metabolism and influence the end product synthesis . Given this in view, nutrients type and their concentrations in the medium play an important role in commencing the production of primary

and secondary metabolites as limited supply of an essential nutrient can restrict the growth of microbial cells or product formation. Generally, carbon and nitrogen sources present in the medium can influence the metabolite production.

Carbon Source

Carbon is the most important medium component, as it is an energy source for the microorganisms and plays an important role in the growth as well as in the production of primary and secondary metabolite. The rate at which the carbon source is metabolized can often influence the formation of biomass and/or the production of primary or secondary metabolites. The antibiotics production from marine bacteria noticed that the gradually assimilating carbon sources, like, galactose generally enhances the production of secondary metabolites (antibiotics). A classic example for this is, penicillin production, where glucose is found to have repression effect. Later, it was found that lactose is a slowly assimilating carbon source and helped in the production of secondary metabolites (i.e., penicillin). Hence, in order to overcome the carbon catabolite repression phenomenon, the production process was established using lactose fermentation. Describing the role of each carbon in different fermentation processes, will increase the length of this manuscript. Hence we compiled a list, wherein we summarized some interfering and non-interfering carbon sources (Table 1).

Table 1. Examples of some interfering and non-interfering carbon sources.

Carbon	Source	Action	Metabolites	Producer	References
Simple carbon	Glycerol	Interfering	Actinomycin D	*Streptomyces parvullus*	Foster and Katz, 1981
			Erythromycins	*Saccharopolyspora erythraea*	Sánchez et al., 2010
			Cephalosporin	*Cephalosporium acremonium*	Sanchez and Demain, 2002
		Non-interfering	Simocyclinones	*Streptomyces antibioticus*Tü 6040	Theobald et al., 2000
Monosaccharide	Glucose	Interfering	Actinomycin	*Streptomyces* sp.	Gallo and Katz, 1972
			Cephalosporin	*Cephalosporium acremonium*	Sanchez and Demain, 2002
			Erythromycins	*Saccharopolyspora erythraea*	Sánchez et al., 2010
			Penicillin	*Streptomyces chrysogenum*	Sanchez and Demain, 2002
			Streptomycin	*Streptomyces griseus*	Sanchez and Demain, 2002
		Non-interfering	Bacilysin	*Bacillus subtilis*	Ozcengiz et al., 1990
	Fructose	Interfering	Penicillin	*Penicillium chrysogenum*	Sanchez and Demain, 2002
		Non-interfering	Actinomycin	*Streptomyces antibioticus*	Rokem et al., 2007
			Gentamycin	*Micromonospora purpurea*	Sanchez and Demain, 2002
	Galactose	Interfering	Penicillin	*Penicillium chrysogenum*	Sanchez and Demain, 2002
		Non-interfering	Actinomycin	*Streptomyces antibioticus*	Rokem et al., 2007
			Cephalosporin	*Cephalosporium acremonium*	Sanchez and Demain, 2002
Disaccharide	Maltose	Interfering	Bacilysin	*Bacillus subtilis*	Ozcengiz et al., 1990
		Non-interfering	Gentamycin	*Micromonospora purpurea*	Sanchez and Demain, 2002
	Sucrose	Interfering	Erythromycins	*Streptomyces erythreus*	Rokem et al., 2007
			Penicillin	*Penicillium chrysogenum*	Sanchez and Demain, 2002
		Non-interfering	Cephalosporin	*Cephalosporium acremonium*	Sanchez and Demain, 2002
	Lactose	Interfering	*		
		Non-interfering	Erythromycins	*Streptomyce serythreus*	Rokem et al., 2007
			Penicillin	*Penicillium chrysogenum*	Rokem et al., 2007
	Mannose	Interfering	Erythromycin	*Streptomyce serythreus*	Sanchez and Demain, 2002
			Streptomycin	*Streptomyces griseus*	Sánchez et al., 2010
		Non-interfering	Kanamycin	*Streptomyces kanamyceticus*	Sanchez and Demain, 2002
Complex	Starch	Interfering	*		
		Non-interfering	Kanamycin	*Streptomyces kanamyceticus*	Rokem et al., 2007

**Not reported.*

Fermentation processes, where raw materials/medium components cover the significant portion of the product cost, selection of these things become an important task for the production companies. In addition to the rate of assimilation of carbon sources, the nature of carbon source also affects the type and amount of the product. An example of this is ethanol or single-cell protein production, where the raw materials contribute ~60–77% of the production cost; and the selling price of the product is determined largely by the cost of the carbon source. Methanol could be a very popular inexpensive carbon source for single-cell protein production, but being toxic to the cells even at low concentrations and low flash points, it can never be used in fermentation as media. Hence, not only the cost even the dynamics of the carbon source must be considered whether it plays a role as a substrate in fermentation process or not.

Nitrogen Source

Like carbon, the selection of nitrogen source and its concentration in the media also play a crucial role in metabolite production. The microorganism can utilize both inorganic and/or organic sources of nitrogen. Use of specific amino acids can increase the productivity in some cases and conversely, unsuitable amino acids may inhibit the synthesis of secondary metabolites. Singh et al. (2009) during the optimization of actinomycin V production by Streptomyces triostinicus found that biosynthesis of actinomycin V involves tryptophan pathway and addition of amino acid tryptophan to the medium enhances the production. On the contrary, the same amino acid showed inhibitory effect in the production of candicidin from *Streptomyces griseus*. Nevertheless, it is confirmed that nitrogen molecules have inhibitory effect on the metabolite production in some cases, whereas, some enhancer effects of nitrogen have also been reported (Table 2).

Table 2. Examples of some interfering and non-interfering nitrogen sources.

Nitrogen	Source	Action	Metabolites	Producer	References
Inorganic	NH_4^+	Interfering	Spiramycin	*Streptomyces ambofaciens*	Lebrihi et al., 1992
			Cephalosporin	*Cephalosporium acremonium*	Sanchez and Demain, 2002
			Erythromycin	*Streptomyces erythreus*	Rokem et al., 2007
			Streptomycin	*Streptomyces griseus*	Sanchez and Demain, 2002
			Tetracycline	*Streptomyces* spp.	Rokem et al., 2007; Vastrad and Neelagund, 2011
		Non-interfering	*		
	Nitrate	Interfering	Aflatoxin	*Aspergillus parasiticus*	Sanchez and Demain, 2002
		Non-interfering	Rifamycin	*Amycolatoposis mediterranei*	Sanchez and Demain, 2002
Organic	Urea	Interfering	Alternariol	*Alternaria alternata*	
		Non-interfering	*		
Amino acids	L-alanine	Interfering	Actinomycin	*Streptomyces antibioticus*	Rokem et al., 2007
			Bacilysin	*Bacillus subtilis*	Ozcengiz et al., 1990
		Non-interfering	*		
	L-arginine	Interfering	*		
		Non interfering	Cephalosporin	*Cephalosporium acremonium*	Sanchez and Demain, 2002
			Gramicidin S	Bacillus brevis	Poirier and Demain, 1981
	d,l-Aspartate	Interfering	Actinomycin D	*Streptomyces parvullus*	Foster and Katz, 1981
		Non-interfering	Streptothricin	*Streptomyces rochei*	Sanchez and Demain, 2002
	Leucine	Interfering	Monascus pigment	*Monascus* spp.	Lin and Demain, 1994
		Non-interfering	Chloramphenicol	*Streptomyces venezuelae*,	Rokem et al., 2007
	L-isoleucine	Interfering	Actinomycin D	*Streptomyces parvullus*	Foster and Katz, 1981
		Non-interfering	Spiramycin	*Streptomyces ambofaciens*	Lebrihi et al., 1992
	DL- phalanine	Interfering	Actinomycin	*Streptomyces antibioticus*	Rokem et al., 2007
		Non-interfering	Chloramphenicol	*Streptomyces venezuelae*,	Rokem et al., 2007
	L-proline	Interfering	Actinomycin D	*Streptomyces parvullus*	Foster and Katz, 1981
		Non-interfering	Streptomycin	*Streptomyces griseus*	Sanchez and Demain, 2002
	Tryptophan	Interfering	Candicidin	*Streptomyces griseus*	Sanchez and Demain, 2002
		Non-interfering	Actinomycin	*Streptomyces parvullus*	Foster and Katz, 1981

*Not reported.

Phosphate

Phosphate is another basic component which is required for the production of phospholipids present in the microbial cell membranes, and for the production of nucleic acids. The amount of phosphate which must be added in the fermentation medium depends upon the composition of the broth and the need of the organism, as well as according to the nature of the desired product. For instance, some cultures will not produce secondary metabolites in the presence of phosphate, e.g., phosphatase, phytases etc. Various secondary metabolites' production such as, actinorhodin, cephalosporin, clavulanic acid, streptomycin, tetracycline, vancomycin etc. is highly influenced by inorganic phosphate concentration present in the production medium. In most cases, lower concentration of phosphate is required for the initiation of the metabolite (antibiotic) production and beyond a certain concentration it suppresses the secondary metabolism and ultimately inhibits the production of primary or secondary metabolite. High phosphate concentration was reported to inhibit the production of teicoplanin, a glycopeptide antibiotic.

From the above description it is clear that changes in carbon or nitrogen sources of the production medium or variation from their optimum required concentration, may affect the nature of the end product or its productivity. Therefore, the production medium with all the required components in appropriate concentration is required for the production of desired metabolite at large scale. In order to standardize the production medium, the concept of medium optimization has emerged.

Need of Medium Optimization

Medium optimization studies are usually carried out in the chemical, food, and pharmaceutical industries, with respect to increase the yield and activity of the desired product. Currently, there is a very little knowledge available about the role of factors, their levels in controlling the metabolite (e.g., antibiotics, acids) production by different strains. In order to enhance the productivity of the metabolites (for e.g., antibiotics etc.), researchers investigated the nutritional requirements for the production of secondary metabolites and found that the nutritional requirements were varying from strain to strain (Shih et al., 2002; Singh et al., 2012). The quantity and quality of nutrients available and the ability to assimilate successfully are the major determinants of microbial nature and its metabolic activity. Hence, during the medium optimization it must be considered that a minimal growth requirement of the microorganism must be fulfilled for obtaining maximum production of

metabolite(s). As the fermentation process progresses into lower-value, higher-volume chemicals, it becomes necessary to maximize the efficiency and minimize the production cost and waste by-products to compete effectively against the traditional methods.

Media Optimization Strategies

During the medium designing and optimization, there are various strategies available which are frequently used to improve the efficiency of the production medium. Figure 2 is a schematic representation of various techniques used in the medium optimization.

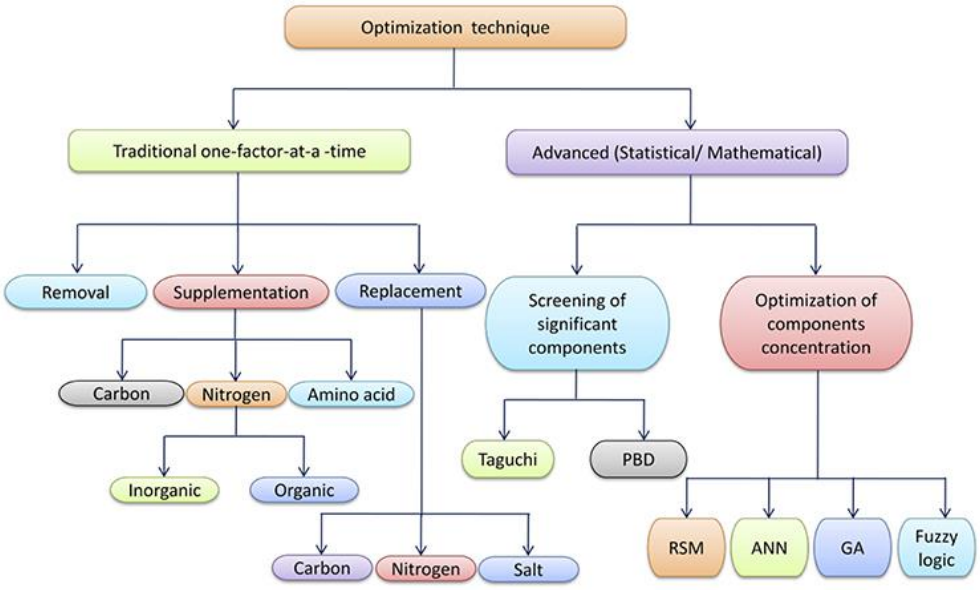


Figure 2. Schematic representation of various techniques used in optimization studies.

Classical Medium Optimization Methods

One-Factor-at-a-Time (OFAT)

In the classical medium optimization technique, one-factor-at-a-time (OFAT) experiments, only one factor or variable is varied at a time while keeping other variables constant. The concentrations of the selected medium components were then changed over a desired range. Because of its ease and convenience, the OFAT has been the most preferred choice among the researchers for designing the medium composition and used in the initial stages in diverse fields (Gonzalez et al., 1995). This methodology is still in use even today, during the initial stages of medium formulation

for the production of new metabolite or known compound from new source. Based upon the approach applied, OFAT is further sub-grouped into:

Removal experiments

In this type of experiment, all the medium components are removed from the production medium one-by-one, and after proper incubation period, their effects on the production of secondary metabolite or the product of interest is observed in terms of suitable parameters. Our research group has previously reported that during the production of antifungal compound from Streptomyces capoamus, removal of soybean meal or glycerol or NaCl from the fermentation medium decreased the yield by 20–40%.

Supplementation experiments

Supplementation experiments are generally performed to evaluate the effects of various carbon and nitrogen supplements on metabolite production. During the study of antifungal production from Streptomyces violaceusniger, 70–90% enhancement in the yield was observed by supplementing xylose, sorbitol and hydroxyl proline in the production medium (Tripathi et al., 2004). Similarly, glycerol and peptone was found as a most suitable carbon and nitrogen sources for the production of antifungal and antibacterial metabolites from Streptomyces rimosus under submerged fermentation condition.

Replacement experiments

For medium formulation, carbon/nitrogen sources showing enhancement effect on the desired metabolite production in supplementation experiments are generally tried to be used as a whole carbon/nitrogen source.

Physical parameters

In addition to chemical and biological variables, several researchers used OFAT experiments to standardize the physical parameters such as pH, temperature, agitation and aeration requirements of the fermentation process.

Like any other technique, OFAT method of medium optimization has its own advantages and disadvantages. The major advantage of OFAT is its simplicity by which a series of experiments can be carried out and results can be analyzed by using simple graphs without the aid of high end statistical analysis/programs. The major drawback of OFAT is the difficulty in estimating the "interactions" from the experiments as it is a hit-and-miss scattershot sequence of the experiments. Vaidya et al. (2003) described the time consumed and cost involved in the analysis of large number of variables as the major disadvantages of OFAT techniques. In this methodology, sometimes the optimum point may be missed completely, thus it requires a large number of experiments to determine the optimum level, which becomes laborious, time consuming, and uneconomical most of the time (Gupte and Kulkarni, 2003). Nevertheless, OFAT technique can be a best screening tool when nothing about the media is known because of its ease and convenience.

Design of Experiments

The use of statistical method, i.e., design of experiments (DOE) for the media optimization in fermentation process can overcome the limitations of classical OFAT method and can be a powerful tool for the optimization of metabolite production. Fisher (1992) proposed a basic theory of experimental design which shows that changing more than one component in the medium at a time can be more efficient over changing only one-factor-at-a-time.

DOE is a series of experiments which are strategically planned and executed to obtain a larger amount of information about the effect of more than one parameter at a time on the output, i.e., product yield. Most DOE procedures allow the preliminary screening of 2–10 medium factors in a limited number of experiments. In this method, several medium factors or components are compared simultaneously and the effects are observed and ranked based on the results. Once the response variables are determined and ranked, statistical performance parameters are generated from the subsequent analysis. Due to the requirement of higher number of experiments, OFAT is laborious, time consuming process, and extremely tedious for a large number of variables, whereas DOE requires fewer experiments, lesser time, and lesser material to obtain the same amount of information. The interaction between the factors can be estimated systematically in DOE. After getting the basic idea about the fermentation production process from the literature or from the

classical experiments, designing of the experiments are more effective to determine the impact of two or more factors on a response than OFAT.

Statistical Medium Optimization

With the advancement of statistical techniques, medium optimization has found new dimensions, as these techniques improve the efficiency of the process, reduces the time required in the process and labor cost etc., thus contributing toward the overall economics of the process. Being, biological in nature, the microbial processes contain relatively large amount of natural variations. The networks associated with the microbial reactions are complex, and several factors affect different parts of the networks. Rational experimental design and statistical evaluation of the results increase the knowledge about the reliability of the information obtained during the experiments. By using experimental design, the amount of experiments required to obtain a for reliable process optimization can be reduced (Elibol, 2004).

Many studies claim substantial improvements over media obtained using OFAT techniques by using DOE methods. For example, during the study of rate of methane and carbon dioxide gas production from Methanosarcina barkeri bacterium growing on methanol, medium optimized through experimental design was found to give 1.3 times more gas production as compared to the OFAT optimized medium (Silveira et al., 1991). Given this, it is widely accepted that in order to have an improved media by employing the experimental design approach; we require both a design as well as the optimization technique. The DOE defines the medium variants to be tested such as, number of replicates and the arrangement of the tests in a harmonized pattern etc. Based upon the obtained experimental data, optimization technique is used to predict a mathematical model and improve the medium composition.

Experimental Design

Experimental design is a study plan to get defined goals or objectives. Modern statistical techniques provide us powerful tools for the evaluation of the components or variables effects based on the experimental results. Hence, the experiments must be planned properly with the sufficient sample size to obtain adequate data which is essential to answer the objective as efficiently as possible. Such types of techniques are commonly called as DOE. In a full factorial

design, all the combinations of the factors, e.g., pH, strain, medium components, temperature etc. are tested. In contrast, in a partial factorial analysis, only few well reported combinations are picked-up and tested. Usually, partial factorial analysis is done, when the full factorial design is not possible and some or little knowledge about the interactions of the medium components for a particular strain is available.

Plackett burman design

All the components present in the medium do not contribute in the metabolite production. Hence, it is utmost important that the non-contributing factors, should be eliminated from the study as early as possible. In 1946, R.L. Plackett and J.P. Burman published their work entitled "The design of optimal multifactorial experiments" as a solution to determine the major effects with higher precision in any process. Plakett Burman Design (PBD), is a two-level design, which is very useful for economically detecting the main effects and assuming all the other interactions are negligible when comparing the some important major effects, i.e., when there are no interactions, the observed effect of a factor can be superior or under estimated by other factors (Vaidya et al., 2003). An example of PBD has been given in Table 3. PDB is used to screen "n" number of experimental variables in just "n+1" number of experiments (Reddy et al., 1999; Ghanem et al., 2000). In this design, there are two types of variables, i.e., "real variables" whose concentration changes during the experiments, and "dummy variables," whose concentration remains constant during the experiments and used to estimate the error. Each variable is represented in two levels, i.e., high (H) and low (L). Each horizontal row represents a trial and each vertical column represents the either of two levels (high or low) of each independent and dummy variables in all the trials. Usually, the classical experiments help in the selection of independent and dummy variables. The effect of each variable is determined by the following equation:

$$Ex1 = 2(\sum Yx_{1H} - \sum Yx_{1L})/N;$$

Where, E(X1) = Effect of variable; YX1-H = yield from the trials having high concentration of variable; YX1−L = yield from the trials having low concentration of variable and N = total number of trials.

Table 3. Plackett-Burman design for eleven variables.

Runs	Variables and levels										
	X1	*X2*	*X3*	*X4*	*X5*	*X6*	*D1*	*D2*	*D3*	*D4*	*D5*
1	L	H	L	L	L	H	H	H	L	H	H
2	H	L	L	L	H	H	H	L	H	H	L
3	L	L	L	H	H	H	L	H	H	L	H
4	L	L	H	H	H	L	H	H	L	H	L
5	L	H	H	H	L	H	H	L	H	L	L
6	H	H	H	L	H	H	L	H	L	L	L
7	H	H	L	H	H	L	H	L	L	L	H
8	H	L	H	H	L	H	L	L	L	H	H
9	L	H	H	L	H	L	L	L	H	H	H
10	H	H	L	H	L	L	L	H	H	H	L
11	H	L	H	L	L	L	H	H	H	L	H
12	L	L	L	L	L	L	L	L	L	L	L

H, high conc. of the components; L, low conc. of the components; D, dummy variable.

Experimental error is estimated by calculating the variance among the dummy variables as follows: Veff = ∑(E2d)/n; where Veff = variance of the concentration effect, Ed = effect for dummy variable and n = number of dummy variables. The standard error (SE) of the concentration effect is the square root of the variance (√ Veff). The significance level of the effect of each variable is determined by student's t-test: tx1 = Ex1/SE. The variables with confidence levels greater than 90–95% will be considered to influence the metabolite production significantly.

PBD is an authentic method to evaluate the relative importance of various variables or medium components for specific output, for e.g., antibiotic or other cellular metabolite production (Ghanem et al., 2000; Vaidya et al., 2003; Singh and Tripathi, 2008; Rajeswari et al., 2014). Use of PBD decreases the total number of experiments, tremendously (Adinarayana and Ellaiah, 2002), as the interaction effects of the variables not consider and only those variables that actually affect the production of desired metabolite are screened. For gamma interferon production using PBD, 20 medium components were examined in only 24 runs, and 45% higher production was observed (Castro et al., 1992). Likewise, during the initial studies of medium optimization for antibacterial metabolite production from Streptomyces sp, we have used PBD to identify the most effective components in the media and reported soybean meal, calcium carbonate, and potassium phosphate can significantly increase the antibiotic production (Banga et al., 2008).

Even though PBD is a good method to identify the important components, but there are some drawbacks associated with its efficiency. PBD should be used only when the factors have no interactions, or have only additive effects on the output, otherwise the results of the factor analyzed will be enhanced or masked by other factors as it fails to interpret if the effect of one factor depends on another factor. Nevertheless, in the DOE, PBD is a starting point and one should use it to determine the follow-up experimentation list. Given this, PBD is usually called “screening designs” because they help you to screen out non-contributing factors, i.e., for higher yield, from that of contributing factors.

INOCULUM DEVELOPMENT

Introduction

The main objective of inoculum development for traditional bacterial fermentations is to decrease lag phase.

A long lag phase is not only is wastage of time but also medium consumed in maintaining a viable culture prior to growth.

The size of the inoculum and its physiological condition affect the length of the lag phase.

Bacterial inocula should transfer when the cells are still metabolically active.

The age of the inoculum is particularly important in the growth of sporulating bacteria, for sporulation induced at the end of the logarithmic phase and the use of an inoculum containing a high percentage of spores would result in a long lag phase in a successive fermentation.

The commercial production of proteases uses five percent inoculum of thermophilic Bacillus in logarithm phase.

A two-stage inoculum development programme of *Bacillus subtilis* used for the production of proteases. Inoculum for a seed fermenter was grown for 1 to 2 days on a solid or liquid medium and then transferred to a seed vessel where the organism was allowed to grow for a further ten generations before transfer to the production stage.

The lag phase in plant fermenters eliminated by using inoculum medium of the same composition as used in the production fermenter and employing large inocula of actively growing seed cultures in the production of bacterial enzymes.

Inoculum development programme at pilot- scale for the production of vitamin B12 from ***Pseudomonas denitrificans*** shown below.

STOCK CULTURE

Lyophilised with skim milk

↓

MAINTENANCE CULTURE

Agar slope incubated 4 days at 28°

↓

SEED CULTURE - FIRST STAGE

2 dm3 flask containing 0.6 dm3 medium inoculated with culture from one slope; incubated with shaking for 48h at 28°

↓

SEED CULTURE - SECOND STAGE

40 - 80 dm3 fermenter containing 25 - 50 dm3 medium inoculated with 1 - 1.2% first stage seed culture. Incubated 25 - 30h at 32°

↓

PRODUCTION CULTURE

500 dm3 fermenter with 300 dm3 medium inoculated with 5% second stage seed culture. Incubated at 32° for 140 - 160 h

The acetic-acid bacteria used in the vinegar process are extremely sensitive to oxygen starvation therefore, it is essential to use an inoculum in an active physiological state.

The cells at the end of fermentation are use as inoculum for the next batch by removing approximately 60% of the culture and restoring the original level with fresh medium.

In this process, there are enough chances of strain degeneration and contaminant accumulation.

However, strain stability is a major concern in inoculum development for fermentations employing recombinant bacteria.

Plasmid stability and productivity in ***E. coli*** biotin fermentation improved if stationary, rather than exponential phase, cells used as inoculum due to loss of plasmid in fermentation.

In the lactic-acid fermentation, lactic acid inhibits the production organism. Thus, production of lactic acid in the seed fermentation may result in generation of poor quality inoculum.

High quality inoculum of ***Lactococcus lactis*** 10-1 on a laboratory scale obtained using electrodialysis, which reduced the lactate in the inoculum and reduced the length of the lag phase in the production fermentation.

Development of Inocula for Anaerobic Bacterial Processes

Clostridial Acetone-Butanol fermentation is anaerobic process.

However, the process was outcompeted by the petrochemical industry but there is still considerable interest in reestablishing the fermentation.

The inoculum development programme described by McNeil and Kristiansen (1986) given as below

Heat-shocked spore suspension inoculated into 150 cm3 of potato glucose medium

↓

Stage 1 culture used as inoculum for 500 cm3molasses medium

↓

Stage 2 culture used as inoculum for 9 dm3 molasses medium

↓

Stage 3 culture used as inoculum for 90,000 dm3 molasses medium

The stock culture is heat shocked to stimulate spore germination and to eliminate the weaker spores.

The production stage inoculated with a very low volume.

The use of such small inocula necessitates the achievement of as near perfect conditions as possible to prevent contamination and to avoid an abnormally long lag phase.

The Development of Inocula for Yeast Processes

Industrial uses of yeasts are

1 The brewing of beer

2 The production of Baker's Yeast (biomass) and

3 For the production ofrecombinant products from the yeast

Brewing

Yeast used to inoculate a fresh batch of wort from previous fermentation or from propagator.

It is common practice in the British brewing industry to use the yeast from the previous fermentation.

The brewing terms used to describe this process and, 'crop' referring to the harvested yeast from the previous fermentation and 'pitch' meaning to inoculate.

One of the major factors contributing to the continuation of this practice is the wort-based excise laws in the United Kingdom where duty charged on the sugar consumed rather than the alcohol produced.

Thus, dedicated yeast propagation systems are expensive to operate because duty charged on the sugar consumed by the yeast during growth.
The problems with this technique are chances of contamination and degeneration of strains the most common problem with the degenerated cell is the change in the degree of flocculence and weakening of abilities of the yeast.

In breweries employing top fermentations in open fermenters, the above dangers minimized by collecting yeast to be used for future pitching from 'middle skimmings''. As the head of yeast develops the surface layer, (the most flocculent and highly contaminated yeasts) removed and discarded and the underlying cells (the 'middle skimmings') harvested and used for subsequent pitching.

Therefore, the 'middle skimmings' contain cells which have the desired flocculence and which have been protected from contamination by the surface layer of the yeast head.
The pitching yeast may be treated to reduce the level of contaminating bacteria and remove protein and dead yeast cells by such treatments as reducing the pH of the slurry to 2.5 to 3, washing with water, washing with ammonium persulphate and treatment with antibiotics such as,polymixin, penicillin and neomycin.
However, traditional open vessels are becoming rare and the bulk of beer brewed using cylindro-conical fermenters.

In these systems, the yeast flocculates and collects in the cone at the bottom of the fermenter where it is subject to the stresses of nutrient starvation, high ethanol concentration, low water activity, high carbon dioxide concentration and high pressure, which decreases the viability and physiological state of the yeast crop, would not be ideal for an inoculum.
The situation is further complicate by the fact, that the harvested yeast is stored rapidly to about one degree before it is used as inoculum suspending in beer and storing in the absence of oxygen.
One of the key physiological features of yeast inoculum is the level of sterol in the cells. Sterols are required for synthesis of membrane but they are only produce in the presence of oxygen.
Thus, we have the irregularity of oxygen being required for sterol synthesis yet anaerobic conditions are required for ethanol production.

This irregularity is resolved traditionally by aerating the wort before inoculation.
The difficulties outlined above and the likelihood of strain degeneration and contamination mean that rarely used for more than five to ten consecutive fermentations that necessitates the periodical production of a pure inoculum.

Pure inocula prepared by a yeast propagation scheme utilizing a ten percent of inoculum volume at each stage in the programme and employing conditions similar to those used during brewing. Continuous aeration used during the propagation stage, which seems to have little effect on the beer produced in the subsequent fermentation.

Yeast inoculum produced in this way would also be sterol rich obviating the need for aerated wort. The simplest type of propagator is a single stage system resembling an unstirred aerated fermenter, which inoculated with a shake-flask culture developed from a single colony.
Two-stage systems propagator operated semi-continuously. It consisted of two linked vessels one point five and one fifty cubic decimeters respectively.
The smaller vessel filled with wort sterilized, cooled, aerated, and inoculated with a flask-grown culture. After growth for three to fourdays, the culture forced by air pressure into the second vessel, which, filled with, sterilized cooled wort and aerated.

After mixing an aliquot of 1.5 dm3in second vessel, it is force back into the first vessel. In a further 3 to 4 days, the larger vessel contained sufficient biomass to pitch a thousand cubic decimeters fermenter and the first vessel contained sufficient inoculum for another second stage. However, although this procedure should produce a pure inoculum there is a danger of strain degeneration occurring in such a semi-continuous system.

Baker's Yeast

The commercial production of bakers' yeast involves the development of an inoculum through a large number of aerobic stages.

Although the production stages, of the process, may not operate under strictly aseptic conditions, a pure culture is use for the initial inoculum thereby keeping contamination to a minimum in the early stages of growth.

The development of inoculum for the production of bakers' yeast involve eight stages the first three being aseptic while the remaining stages were carried out in open vessels.
The yeast pumped from one stage to the next or the seed cultures may be centrifuge and washed before transfer, which reduces the level of contamination.

The yields obtained in the first five stages are relatively low because they are not fed-batch systems whereas the last three stages are fed-batch

Types Of Fermentations

Fermentation types are classified into different classes based on various aspects like based on feeding substrate to fermenter, based on need of supply of aeration, based on need of light etc.

1. Based on feeding substrate to fermenter

a. Batch fermentation

b. Continuous fermentation

c. Fed-batch fermentation.

a. In batch fermentation, every material for process of fermentation including substrate, inoculum and all the process parameters are set and filled in a fermenter and the process is set on and until the total process comes to an end neither substrate is added into fermenter nor product is taken out of fermenter. Its a closed system.

b. In continuous fermentation, the substrate is added continuously to the fermenter at a fixed rate which maintains the microbes at logarithmic growth phase and the products that are formed are taken out simultaneously and here we find growth associated products.

c. In fed-batch mode we find both modes of operations of batch and continuous modes, where substrate is added at fixed time intervals during the fermentation process.

2. Based on need of supply of aeration

a. aerobic fermentation,

b. anaerobic fermentation

a. Aerobic fermentations: even though called fermentation, many large-scale fermentation processes are carried out in presence of aerobic conditions where, the contents present in fermenter are agitated with the help of agitator and with the help of spargers by forcing sterilized air into the fermenter.

b. Anaerobic fermentation: apart from intense need and presence of agitator and sparger to supply aeration, rest of the configuration of the fermenter is as same as aerobic fermentation. But the presence of agitator is made compulsion for the even distribution of temperature, pH, viscosity, nutrients etc. along the medium in the fermenter. Anyways, micro aerophilic conditions to anaerobic conditions are needed to initiate the growth of the microbe in the fermenter. 3. Based on need of light

a. photo fermentation (only photosynthetic bacteria can undergo),

b. Dark fermentation.

a. Photofermentation: its a process of conversion of organic substances to other utilisable energy compounds following a series of biochemical reactions carried out by a specific group of bacteria named Photosynthetic bacteria, which only proceeds in the presence of light.
b. Dark fermentation: in every way dark fermentation is similar to that of photofermentation, but in aspect of need of light, dark fermentation does not need any light to initiate the reactions and a diversified group of microbes are involved in dark fermentation.

Design of a Fermentor

Fermentation

Fermentation can be defined as a metabolic process in which cheap raw materials such as sugar or carbohydrates are converted into economically important products like acids, gases and alcohols by micro-organism. This process is carried out in a equipment called as fermentor.
Fermentation Technology could be defined simply as the study of the fermentation process, techniques and its application. Fermentation should not be seen merely as a process that is entirely focused on the happenings occurring in the fermenter alone! There are many activities that occur upstream leading to the reactions that occur within the bioreactor or fermenter, despite the fermenter is regarded as the heart of the fermentation process.

Fermentation technology is the whole field of study which involves studying, controlling and optimization of the fermentation process right up from upstream activities, mid stream and downstream or post fermentation activities.

The study of fermentation technology requires essential inputs from various disciplines such as biochemistry, microbiology, genetics, chemical and bioprocess engineering and even a scatter of mathematics and physics.

Fermentation in terms of biochemistry and physiology:-

Fermentation is now defined as a process of energy generation by various organisms especially microorganisms. The fermentation process showed unique characteristics by which it generates energy in the absence of oxygen. The process of energy generation utilizes the use of substrate

level phosphorylation (SLP) which do not involved the use of electron transport chain and free oxygen as the terminal electron acceptor.

Engineers definition of fermentation:-

It is only up to recently with the rise of industrial microbiology and biotechnology that the definition of fermentation took a less specific meaning. Fermentation is defined more from the point of view of engineers. They see fermentation as the cultivation of high amount of microorganisms and biotransformation being carried out in special vessels called fermenter or bioreactors.

Their definitions make no attempt to differentiate whether the process is aerobic or anaerobic. Neither are they bothered whether it involves microorganisms or single animal or plant cells.

They view bioreactors as a vessel which is designed and built to support high concentration of cells.

A bioreactor is a specially designed vessel which is built to support the growth of high concentration of microorganisms. It must be so designed that it is able to provide the optimum environments or conditions that will allow supporting the growth of the microorganisms.

Bioreactors are commonly cylindrical vessels with hemispherical top and/or bottom, ranging in size from some liter to cube meters, and are often made of stainless steel and glass.

The difference between a bioreactor and a typical composting system is that more parameters of the composting process can be measured and controlled in bioreactors.

The sizes of the bioreactor can vary over several orders of magnitudes. The microbial cell (few mm^3), shake flask (100-1000 ml), laboratory fermenter (1 – 50 L), pilot scale (0.3 – $10m^3$) to plant scale (2 – 500 m^3) are all examples of bioreactors.

The design and mode of operation of a fermenter mainly depends on the production organism, the optimal operating condition required for target product formation, product value and scale of production. The design also takes into consideration the capital investment and running cost.

- Large volume and low value products like alcoholic beverages need simple fermenter and do not need aseptic condition.
- High value and low volume products require more elaborate system of operation and aseptic condition.

Bioreactors differ from conventional chemical reactors in that they support and control biological entities. As such, bioreactor systems must be designed to provide a higher degree of control over process upsets and contaminations, since the organisms are more sensitive and less stable than Chemicals. Biological organisms, by their nature, will mutate, which may alter the biochemistry of the bioreaction or the physical properties of the organism. Analogous to heterogeneous catalysis, deactivation or mortality occur and promoters or coenzymes influence the kinetics of the bioreaction. Although the majority of fundamental bioreactor engineering and design issues are similar, maintaining the desired biological activity and eliminating or minimizing undesired activities often presents a greater challenge than traditional chemical reactors typically require.

Other key differences between chemical reactors and bioreactors are selectivity and rate. In bioreactors, higher selectivity — that is, the measure of the system's capability for producing the preferred product (over other outcomes) — is of primary importance. In fact, selectivity is especially important in the production of relatively complex molecules such as antibiotics, steroids, vitamins, proteins and certain sugars and organic acids. Frequently, the activity and desired selectivity occur in a substantially smaller range of conditions than are present in conventional chemical reactors. Further, deactivation of the biomass often poses more severe consequences than a chemical upset.

The Designing of a Bioreactor also has to take into Considerations the Unique Aspects of Biological Processes

a. The concentrations of starting materials (substrates) and products in the reaction mixture are frequently low; both the substrates and the products may inhibit the process. Cell growth, the structure of intracellular enzymes, and product formation depend on the nutritional needs of the cell (salts, oxygen) and on the maintenance of optimum biological conditions (temperature, concentration of reactants, and pH) within narrow limits.

b. Certain substances inhibitors effectors, precursors, metabolic products influence the rate and the mechanism of the reactions and intracellular regulation.

c. Microorganisms can metabolize unconventional or even contaminated raw materials (cellulose, molasses, mineral oil, starch, wastewater, exhaust air, biogenic waste), a process which is frequently carried out in highly viscous, non-Newtonian media.

d. In contrast to isolated enzymes or chemical catalysts, microorganisms adapt the structure and activity of their enzymes to the process conditions, whereby selectivity and productivity can change. Mutations of the microorganisms can occur under sub optimal biological conditions.

e. Microorganisms are frequently sensitive to strong shear stress and to thermal and chemical influences.

f. Reactions generally occur in gas-liquid -solid systems, the liquid phase usually being aqueous.

g. The microbial mass can increase as biochemical conversion progresses. Effects such as growth on the walls, flocculation, or autolysis of microorganisms can occur during the reaction.

h. Continuous bioreactors often exhibit complicated dynamic behaviour.

Requirements of Bioreactors

Due to above mentioned demands made by biological systems on their environment, there is no universal bioreactor. However, the general requirements of the bioreactor are as follows:

1. The vessel should be robust and strong enough to withstand the various treatments required such as exposure to high heat, pressure and strong chemicals and washings and cleanings.
2. The vessel should be able to be sterilized and to maintain stringent aseptic conditions over long periods of the actual fermentation process.
3. The vessel should be equipped with stirrers or mixers to ensure mass transfer processes occur efficiently.
4. It should have sensors to monitor and control the fermentation process.
5. It should be provided with inoculation point for aseptic transfer in inoculum.
6. Sampling valve for withdrawing a sample for different tests.
7. Baffles should be provided in case of stirred fermenter to prevent vertex formation.
8. It should be provided with facility for intermittent addition of an antifoam agent.
9. In case of aerobic submerged fermentation, the tank should be equipped with the aerating device.
10. Provision for controlling temperature and pH of fermentation medium.
11. Man hole should be provided at the top for access inside the fermenter for different purposes.

Fermenter Design

What should be the basic points of consideration while designing a fermenter?

- Productivity and yield
- Fermenter operability and reliability
- Product purification
- Water management
- Energy requirements
- Waste treatment

Few Significant things of concern that should be taken into account while designing a fermenter:

- Design in features so that process control will be possible over reasonable ranges of process variables.
- Operation should be reliable
- Operation should be contamination free.
- Traditional design is open cylindrical or rectangular vessels made from wood or stone.
- Most fermentation is now performed in close system to avoid contamination.
- Since the fermenter has to withstand repeated sterilization and cleaning, it should be constructed from non-toxic, corrosion-resistant materials.
- Small fermentation vessels of a few liters capacity are constructed from glass and/or stainless steel.
- Pilot scale and many production vessels are normally made of stainless steel with polished internal surfaces.
- Very large fermenter is often constructed from mild steel lined with glass or plastic, in order to reduce the cost.
- If aseptic operation is required, all associated pipelines transporting air, inoculum and nutrients for the fermentation need to be sterilizable, usually by steam.
- Most vessel cleaning operations are now automated using spray jets, which are located within the vessels. They efficiently disperse cleaning fluids and this cleaning mechanism is referred to as cleaning-in-place CIP.

Associated pipe work must also be designed to reduce the risk of microbial contamination.

There should be no horizontal pipes or unnecessary joints and dead stagnant spaces where material can accumulate; otherwise this may lead to ineffective sterilization. Overlapping joints are unacceptable and flanged connections should be avoided as vibration and thermal expansion can result in loosening of the joints to allow ingress of microbial contaminants.

- Butt welded joints with polished inner surfaces are preferred.

Normally, fermenters up to 1000 L capacity have an external jacket, and larger vessels have internal coils. Both provide a mechanism for vessel sterilization and temperature control during the fermentation.

Other features that must be incorporated are pressure gauges and safety pressure valves, which are required during sterilization and operation. The safety valves prevent excess pressurization, thus reducing potential safety risks. They are usually in the form of a metal foil disc held in a holder set into the wall of the fermenter. These discs burst at a specified pressure and present a much lower contamination risk than spring-loaded valves.

For transfer of media pumps are used. However pumps should be avoided if aseptic operation is required, as they can be a major source of contamination. Centrifugal pumps may be used, but their seals are potential routes for contamination. These pumps generate high shear forces and are not suitable for pumping suspensions of shear sensitive cells. Other pumps used include magnetically coupled, jet and peristaltic pumps.

Alternate methods of liquid transfer are gravity feeding or vessel pressurization. In fermentations operating at high temperatures or containing volatile compounds, a sterilizable condenser may be required to prevent evaporation loss. For safety reasons, it is particularly important to contain any aerosols generated within the fermenter by filter- sterilizing the exhaust gases.

Also, fermenters are often operated under positive pressure to prevent entry of contaminants.

2. <u>Considerations that improve productivity of bioreactor:</u> -

2.1 Material of construction: -

2.1.1 Laboratory scale bioreactor:

In fermentation with strict aseptic requirements it is important to select materials that can withstand repeated sterilization cycles. On a small scale, it is possible to use glass and/or stainless steel.

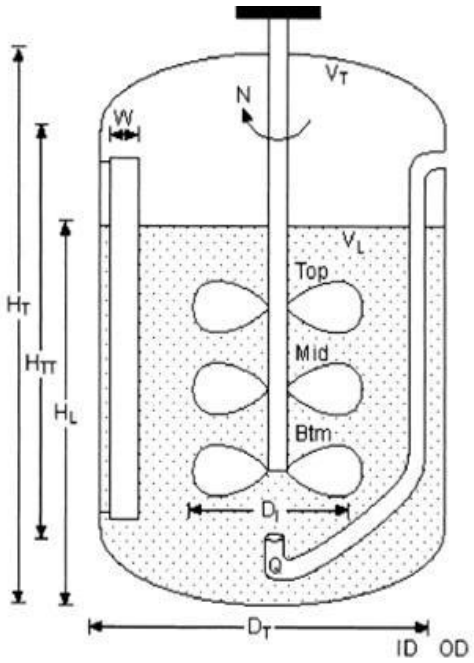
Glass is useful because it gives smooth surfaces, is non-toxic, corrosion proof and it is usually easy to examine the interior of vessel. The glass should be 100% borosilicate, e.g. Pyrex® and Kimax®.

The following variants of the laboratory bioreactor can be made:

1. Glass bioreactor (without the jacket) with an upper stainless steel lid.

2. Glass bioreactor (with the jacket) with an upper stainless steel lid.
3. Glass bioreactor (without the jacket) with the upper and lower stainless steel lids.
4. Two-part bioreactor - glass/stainless steel.
   The stainless steel part has a jacket and ports for electrodes installation.
5. Stainless steel bioreactor with peepholes.

Vessels with two stainless steel plates cost approximately 50% more than those with just a top plate. The geometrical term of the bioreactors are shown in the figure on the next page.



Schematic of fermentor showing nomenclature.

2.3. Agitation: -

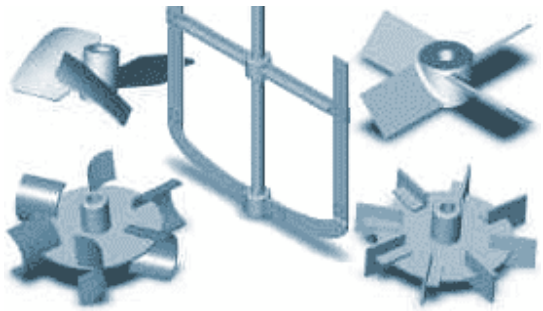
2.3.1 The agitator (impeller).

The agitator is required to achieve a number of mixing objective.

- Bulk fluid and gas-phase mixing,
- Air dispersion,
- Oxygen transfer,
- Heat transfer,
- Suspension of solid particles and maintain a uniform environment throughout the vessel contents.
- Enhancement of mass transfer between dispersed phase

Bulk mixing and micro mixing both are influenced strongly by impeller type, broth rheology, and tank geometry and internals.

Impellers used bioreactors are:



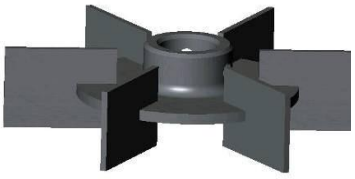
Rushton disc turbines, vaned discs, open turbines of variable pitch and propeller. The disc turbine consists of a disc with a series of rectangular vanes set in vertical plane around the circumstances and vaned disc has a series of rectangular vanes attached vertically to the underside. Air from the sparger hits the underside of the disc and is displaced towards the vanes where the air bubbles are broken up into smaller bubbles. The vanes of variable pitch open turbine and the blade of marine impellers are attached directly to a boss on the agitator shaft. In this case air bubbles do not initially hit any surface before dispersion by the vanes or blades.

The Rushton disc turbine is the one used most often for highly aerobic fermentations, because it has among the highest power draws of any the commercially available impellers, and it is better characterized than others; hence, its behaviour is easier to predict. Rushton disc turbine of one third of the fermenter diameter has been considered the optimum design for use in fermentation processes. Disc turbine is most suitable in a fermenter since it can break up a fast air stream without itself becoming flooded in air bubbles. A marine propeller is an axial flow impeller which provides good top-to-bottom mixing. It is low power device does not provide large oxygen-transfer rates.

The propeller and the open turbine flood when superficial velocity (Vs) exceeds 21 m h^{-1} , whereas the flat blade turbine can tolerate Vs of 120 m h^{-1} before being flooded, when two sets are used on the same shaft. Besides this, propeller is also less efficient in breaking up the bubbles and the flow it produces is axial rather than the radial. One of major drawback of Rushton disc turbine is that it

provides very axial flow, resulting in poor overall top-to-bottom mixing. In addition, agitation intensity decrease with distance from the impeller, and this decrease can become more pronounced for viscous, pseudoplastic broths

2.3.2 Various impellers use in bioreactors with their flow patterns.

Flat blade disk turbine	**45° Flat blade disk turbine**	**Curved blade disk turbine**
		

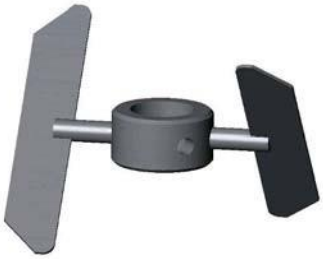
Pitched blade turbine

Marine propeller

Intermig

curved blade turbine

Large pitch blade impeller



3 segment blade impeller

Temperature Control

During the fermentation process heat can be produced mainly in two ways, firstly microbial biochemical reactions and secondly mechanical agitation. In case of fermentation, a temperature control helps to control the temperature at the optimum level by removing or providing heat. In small scale production vessel the amount of produced heat is negligible. Therefore, extra heat is provided by hot bath or internal heat coil or heating jacket with a water circulation system or silicon heating jacket. The silicon heating jacket consists of silicon rubber mats with heating wires and it is wrapped around the fermenter. In the case of pilot-scale fermenters, it is not possible to use silicon jackets due to large size. In such cases, an internal heating coil is used for providing extra heat while cold water circulation helps to remove excess heat.

Stirrer glands and bearings

The most important factor of designing a fermenter is to maintain aseptic conditions inside the vessel. It is highly challenging in the case of pilot-scale fermenters. Therefore stirrer shafts are required. These stirrer shafts play an important role to seal the openings of a bioreactor. As a result, it restricts the entry of air from outside. There are several types of seals used for this purpose, which are following

The Stuffing Box: The shafted is sealed by several layers of packing rings of asbestos or cotton yarn which is pressed against the shaft by gland follower. At high stirrer speeds, the packing wears quickly and excessive pressure may need to ensure the tightness of fit. The packing may be difficult to sterilize properly because of unsatisfactory heat penetration and it is necessary to check and replace the packing rings regularly.

The Mechanical Seal: It is used in both small scale and large scale fermenters. The seal is divided into two parts, first is the stationary bearing housing and the second rotates on the shaft. These two parts are pressed together by springs. Steam condensate is used to lubricate and cool the seals during operation and provides protection against the contamination.

Magnetic Drives: This type of seals helps to counter the problem originated by the impeller shaft which is going through the top or bottom of the fermenter plate. The magnetic drive is made up of two magnets one is driving and one driven. The driven magnet held in bearings in housing on the outside of the head plate and connected to a drive shaft. The internal driven magnet is placed on one end of the impeller shaft and held in bearings in a suitable housing on the inner surface of the head plate. When multiple ceramic magnets have been used it has been possible to transmit power across a gap of 16mm. Using this drive water can be stirred in baffled vessels up to 300 dm3 capacity at speeds of 300 to 2000 rpm.

Baffles

There are four baffles that are present inside of an agitated vessel to prevent a vortex and improve aeration efficiency. Baffles are made up of metal strips roughly one-tenth of the vessel diameter and attached to the wall. The agitation effect is slightly increased with wider baffles but drops sharply with narrower baffles. After installation of the baffle there a gap between them and the vessel wall which facilitates scouring action around the baffles and minimizes microbial growth

on the baffles and the fermenter wall. Baffles are often attached to cooling coils to increase the cooling capacity of the fermenter.

The aeration system (sparger)

A sparger is a device that introduces air into the liquid medium in a fermenter. There are three main types of fermenter used in industrial-scale bioreactors such as

Porous Sparger: It is made up of sintered glass, ceramics or metals' and are mostly used in laboratory-scale bioreactors. As it introduces air inside a liquid medium, bubbles are formed. These bubbles are always 10 to 100 times larger than the pore size of the aerator. The air pressure is generally low in these devices and a major disadvantage of using porous sparger is that microbial growth may occur on the pores which hamper the airflow.

Orifice Sparger: These are used in small stirred fermenters where perforated pipes are used and attached below the impeller in the form of a ring. The air holes are mostly drilled under the surface of the tubes. Orifice spargers were used to a limited extent in yeast manufacture, effluent treatment and production of single-cell proteins.

Nozzle Sparger: This is used in industrial-scale fermenters. The main characteristic of this kind of sparger is that it contains a single open or partially closed pipe as an air outlet. The pipe needs to be positioned below the impeller. The design helps to overcome troubles related to sparger blockage.

pH control sensors

All types of fermenters are attached with a pH control sensor which consists of a pH sensor and a port to maintain the pH inside of the fermenter. pH alteration can lead to death of the organism which leads to product loss. Therefore, it is a crucial instrument for a fermenter and needs to be checked regularly.

Media

A growth medium or culture medium is a solid, liquid or semi-solid designed to support the growth of a population of microorganisms or cells via the process of cell proliferation, or small plants like the moss Physcomitrella patens. Different types of media are used for growing different types of cells.

The two major types of growth media are those used for cell culture, which use specific cell types derived from plants or animals, and microbiological culture, which are used for growing microorganisms, such as bacteria or fungi. The most common growth media for microorganisms are nutrient broths and agar plates; specialized media are sometimes required for microorganism and cell culture growth. Some organisms, termed fastidious organisms, require specialized environments due to complex nutritional requirements. Viruses, for example, are obligate intracellular parasites and require a growth medium containing living cells.

Types

US Food and Drug Administration scientist tests for Salmonella

The most common growth media for microorganisms are nutrient broths (liquid nutrient medium) or lysogeny broth medium. Liquid media are often mixed with agar and poured via a sterile media dispenser into Petri dishes to solidify. These agar plates provide a solid medium on which microbes may be cultured. They remain solid, as very few bacteria are able to decompose agar (the exception being some species in the genera: Cytophaga, Flavobacterium, Bacillus, Pseudomonas, and Alcaligenes). Bacteria grown in liquid cultures often form colloidal suspensions.

The difference between growth media used for cell culture and those used for microbiological culture is that cells derived from whole organisms and grown in culture often cannot grow without the addition of, for instance, hormones or growth factors which usually occur in vivo. In the case of animal cells, this difficulty is often addressed by the addition of blood serum or a synthetic serum replacement to the medium. In the case of microorganisms, no such limitations exist, as they are often unicellular organisms. One other major difference is that animal cells in culture are often grown on a flat surface to which they attach, and the medium is provided in a liquid form, which covers the cells. In contrast, bacteria such as Escherichia coli may be grown on solid or in liquid media.

An important distinction between growth media types is that of defined versus undefined media. A defined medium will have known quantities of all ingredients. For microorganisms, they consist of providing trace elements and vitamins required by the microbe and especially defined carbon and nitrogen sources. Glucose or glycerol are often used as carbon sources, and ammonium salts

or nitrates as inorganic nitrogen sources. An undefined medium has some complex ingredients, such as yeast extract or casein hydrolysate, which consist of a mixture of many chemical species in unknown proportions. Undefined media are sometimes chosen based on price and sometimes by necessity – some microorganisms have never been cultured on defined media.

A good example of a growth medium is the wort used to make beer. The wort contains all the nutrients required for yeast growth, and under anaerobic conditions, alcohol is produced. When the fermentation process is complete, the combination of medium and dormant microbes, now beer, is ready for consumption. The main types are

Cultural media
Minimal media
Selective media
Differential media
Transport media
Indicator media
Culture media
Culture media contain all the elements that most bacteria need for growth and are not selective, so they are used for the general cultivation and maintenance of bacteria kept in laboratory culture collections.
Physcomitrella patens plants growing axenically on agar plates (Petri dish, 9 cm diameter)
An undefined medium (also known as a basal or complex medium) contains:
a carbon source such as Glucose water various salts a source of amino acids and nitrogen (e.g., beef, yeast extract)
This is an undefined medium because the amino-acid source contains a variety of compounds with the exact composition being unknown.
A defined medium (also known as chemically defined medium or synthetic medium) is a medium in which all the chemicals used are known no yeast, animal, or plant tissue is present
Some examples of nutrient media include:
Plate count agar
Nutrient agar

Trypticase soy agar

Minimal media

A defined medium that has just enough ingredients to support growth is called a "minimal medium". The number of ingredients that must be added to a minimal medium varies enormously depending on which microorganism is being grown. Minimal media are those that contain the minimum nutrients possible for colony growth, generally without the presence of amino acids, and are often used by microbiologists and geneticists to grow "wild-type" microorganisms. Minimal media can also be used to select for or against recombinants or exconjugants.

Minimal medium typically contains:

a carbon source, which may be a sugar such as glucose, or a less energy-rich source such as succinate

various salts, which may vary among bacteria species and growing conditions; these generally provide essential elements such as magnesium, nitrogen, phosphorus, and sulfur to allow the bacteria to synthesize protein and nucleic acids

water

Supplementary minimal media are minimal media that also contains a single selected agent, usually an amino acid or a sugar. This supplementation allows for the culturing of specific lines of auxotrophic recombinants.

Selective media

Blood-free, charcoal-based selective medium agar (CSM) for isolation of Campylobacter

Blood agar plates are often used to diagnose infection. On the right is a positive Staphylococcus culture; on the left is a positive Streptococcus culture.

Selective media are used for the growth of only selected microorganisms. For example, if a microorganism is resistant to a certain antibiotic, such as ampicillin or tetracycline, then that antibiotic can be added to the medium to prevent other cells, which do not possess the resistance, from growing. Media lacking an amino acid such as proline in conjunction with E. coli unable to

synthesize it were commonly used by geneticists before the emergence of genomics to map bacterial chromosomes.

Selective growth media are also used in cell culture to ensure the survival or proliferation of cells with certain properties, such as antibiotic resistance or the ability to synthesize a certain metabolite. Normally, the presence of a specific gene or an allele of a gene confers upon the cell the ability to grow in the selective medium. In such cases, the gene is termed a marker.

Selective growth media for eukaryotic cells commonly contain neomycin to select cells that have been successfully transfected with a plasmid carrying the neomycin resistance gene as a marker. Gancyclovir is an exception to the rule, as it is used to specifically kill cells that carry its respective marker, the Herpes simplex virus thymidine kinase.

Four types of agar plate demonstrating differential growth depending on bacterial metabolism

Examples of selective media include:

Eosin methylene blue contains dyes that are toxic for Gram-positive bacteria. It is the selective and differential medium for coliforms.

YM (yeast extract, malt extract agar) has a low pH, deterring bacterial growth.

MacConkey agar is for Gram-negative bacteria.

Hektoen enteric agar is selective for Gram-negative bacteria.

HIS-selective medium is a type cell culture medium that lacks the amino acid histidine.

Mannitol salt agar is selective for Gram-positive bacteria and differential for mannitol.

Xylose lysine deoxycholate is selective for Gram-negative bacteria.

Buffered charcoal yeast extract agar is selective for certain Gram-negative bacteria, especially Legionella pneumophila.

Baird–Parker agar is for Gram-positive staphylococci.

Sabouraud's agar is selective to certain fungi due to its low pH(5.6) and high glucose concentration(3-4%)

DRBC (Dichloran Rose Bengal Chloramphenicol agar) is a selective medium for the enumeration of moulds and yeasts in foods. Dichloran and rose bengal restricts the growth of mould colonies thus preventing overgrowth of luxuriant species and assisting accurate counting of colonies.

Differential media

UTI Agar is a chromogenic medium for differentiation of main microorganisms that cause urinary tract infections (UTIs).

Differential or indicator media distinguish one microorganism type from another growing on the same medium. This type of media uses the biochemical characteristics of a microorganism growing in the presence of specific nutrients or indicators (such as neutral red, phenol red, eosin y, or methylene blue) added to the medium to visibly indicate the defining characteristics of a microorganism. These media are used for the detection of microorganisms and by molecular biologists to detect recombinant strains of bacteria.

Examples of differential media include:

Blood agar (used in strep tests) contains bovine heart blood that becomes transparent in the presence of β-hemolytic organisms such as Streptococcus pyogenes and Staphylococcus aureus.

Eosin methylene blue is differential for lactose fermentation.

Granada medium is selective and differential for Streptococcus agalactiae (group B streptococcus) which grows as distinctive red colonies in this medium.

MacConkey agar is differential for lactose fermentation.

Mannitol salt agar is differential for mannitol fermentation.

X-gal plates are differential for lac operon mutants.

Transport media

Transport media should fulfill these criteria:

Temporary storage of specimens being transported to the laboratory for cultivation

Maintain the viability of all organisms in the specimen without altering their concentration

Contain only buffers and salt

Lack of carbon, nitrogen, and organic growth factors so as to prevent microbial multiplication

Transport media used in the isolation of anaerobes must be free of molecular oxygen.

Examples of transport media include:

Thioglycolate broth is for strict anaerobes.

Stuart transport medium is a non-nutrient soft agar gel containing a reducing agent to prevent oxidation, and charcoal to neutralize.

Certain bacterial inhibitors are used for gonococci, and buffered glycerol saline for enteric bacilli.

Venkataraman Ramakrishna (VR) medium is used for V. cholerae

Enriched media

Enriched media contain the nutrients required to support the growth of a wide variety of organisms, including some of the more fastidious ones. They are commonly used to harvest as many different types of microbes as are present in the specimen. Blood agar is an enriched medium in which nutritionally rich whole blood supplements the basic nutrients. Chocolate agar is enriched with heat-treated blood (40–45 °C), which turns brown and gives the medium the color for which it is named

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SCHOOL OF BIO AND CHEMICAL ENGINEERING

DEPARTMENT OF BIOTECHNOLOGY

UNIT – III– Bioprocess Engineering -1 – SBT1301

INTRODUCTION

A fermentation product is produced by the culture of a certain organism, or animal cell line, in a nutrient medium. If a foreign microorganism invades the fermentation then the following consequences may occur:

1. The medium would have to support the growth of both the production organism and the contaminant, resulting in a loss of productivity.

2. If the fermentation is a continuous one then the contaminant may "outgrow" the production organism and displace it from the fermentation.

3. The foreign organism may contaminate the final product, for example, singlecell protein where the cells, separated from the broth, constitute the product.

4. The contaminant may produce compounds that make subsequent extraction of the final product difficult.

5. The contaminant may degrade the desired product; this is common in bacterial contamination of antibiotic fermentations where the contaminant would have to be resistant to the normal inhibitory effects of the antibiotic and degradation of the antibiotic is a common resistance mechanism, for example, the degradation of β-lactam antibiotics by β-lactamase-producing bacteria.

6. Contamination of a bacterial fermentation with phage could result in the lysis of the culture.

Avoidance of contamination may be achieved by:

1. Effective design and construction of the fermentation plant.

2. Using a pure inoculum to start the fermentation,

3. Sterilizing the medium to be employed.

4. Sterilizing the fermenter vessel.

5. Sterilizing all materials to be added to the fermentation during the process, for example, air, nutrient feeds, antifoams, and pH titrants.

6. Maintaining aseptic conditions during the fermentation.

7. Putting in place detailed operating procedures for sterilization, aseptic maintenance, and staff training.

The extent to which these procedures are adopted is determined by the likely probability of contamination and the nature of its consequences. Some fermentations are described as "protected"—that is, only a very limited range of microorganisms may utilize the medium, or the

growth of the process organism may result in the development of selective growth conditions, such as a reduction in pH. The brewing of beer falls into this category; hop resins tend to inhibit the growth of many microorganisms and the growth of brewing yeasts tends to decrease the pH of the medium. Thus, brewing worts are boiled, but not necessarily sterilized, and the fermenters are thoroughly cleaned with disinfectant solution but are not necessarily sterile. Also, the precautions used in the development of inoculum for brewing are far less stringent than, for example, in an antibiotic fermentation. However, the vast majority of fermentations are not "protected" and, if contaminated, would suffer some of the consequences previously listed. A contaminated "classical" microbial fermentation would only be deemed suitable for down-stream processing, if it conformed with the characteristics described by Stockbridge et al. (2001).

These authors considered the nature of the contaminant, the level of contamination, its ability to survive down-stream processing and the likelihood of the contaminant being a potential pathogen or producing a toxin (Fig. 5.1). Junker, Lester, Brix, Wong, and Nuechterlein (2006) reported that fermentations contaminated by a fungus are normally discarded due to the high probability of fungi producing toxic products. At the other end of the spectrum from protected fermentations are those processes (either microbial or cell culture) producing injectable biologics or vaccines. If contamination is detected in these processes then the batch is rejected, the equipment shut down and an investigation into the cause of contamination initiated (Pollard, 2011). Animal cell cultures are particularly susceptible to contamination due to their low growth rate, and hence the duration of the inoculum development programme as well as the production fermentation. Such processes are also vulnerable to viral and mycoplasma infection. Mycoplasmas lack cell walls and can quickly dominate an animal cell culture, producing up to 106–107 colony forming units (CFU) cm−3 but contributing virtually no turbidity to the culture (Stacey & Stacey, 2000). In a wide-reaching review of aseptic operation, Pollard (2011) summarized the microbial taxa causing contamination of fermentations and their likely origin. The most common contaminants are Gram-positive spore-forming rods (*Bacillus* spp.) usually linked with inadequate medium sterilization, due to the presence of large insoluble medium particles, or poor cleaning of the fermenter prior to sterilization resulting in accumulation of dried broth in crevices and joints. The presence of Gram-negative rods is indicative of cooling water leaks, water in the inlet air, or inadequate filter sterilization while contamination by Gram-positive cocci is usually linked with a failure in air filtration. The economically acceptable frequency of contamination has been cited as one fermentation in a hundred (Sharma & Gurtu, 1993).

MEDIUM STERILIZATION

Media may be sterilized by filtration, radiation, ultrasonic treatment, chemical treatment, or heat. However, for practical reasons, steam is used almost universally for the sterilization of fermentation media. The major exception is the use of filtration for the sterilization of media for animal-cell culture—such media are completely soluble and contain heat labile components making filtration the method of choice. Filtration techniques will be considered later in this chapter. Before the techniques that are used for the steam sterilization of culture media are discussed, it is necessary to discuss the kinetics of sterilization. The destruction of microorganisms by steam (moist heat) may be described as a firstorder chemical reaction and, thus, may be represented by the following equation:

$$-\frac{dN}{dt} = kN$$

where N is the number of viable organisms present, t is the time of the sterilization treatment (minutes), k is the reaction rate constant of the reaction, or the specific death rate (min^{-1}). It is important at this stage to appreciate that we are considering the total number of organisms present in the volume of medium to be sterilized, *not* the concentration— the minimum number of organisms to contaminate a batch is one, regardless of the volume of the batch. On integration of Eq. (5.1), the following expression is obtained:

$$\frac{N_t}{N_0} = e^{-kt} \qquad (5.2)$$

where $N0$ is the number of viable organisms present at the start of the sterilization treatment, Nt is the number of viable organisms present after a treatment period, t minutes.

On taking natural logarithms, Eq. (5.2) is reduced to:

$$\ln\left(\frac{N_t}{N_0}\right) = -kt \qquad (5.3)$$

The graphical representations of Eqs. (5.1) and (5.3) are illustrated in Fig. 5.2, from which it may be seen that viable organism number declines exponentially over the treatment period. A plot of the natural logarithm of $Nt/N0$ against time yields a straight line, the slope of which equals-k. This kinetic description makes two predictions that appear anomalous:

1. An infinite time is required to achieve sterile conditions (ie, $Nt = 0$).

2. After a certain time, there will be less than one viable cell present.

Thus, in this context, a value of *Nt* of less than one is considered in terms of the probability of an organism surviving the treatment. For example, if it was predicted that a particular treatment period reduced the population to 0.1 of a viable organism, this implies that the probability of one organism surviving the treatment is one in ten. This may be better expressed in practical terms as a risk of one batch in ten becoming contaminated. This aspect of contamination will be considered later. The relationship displayed in Fig. 5.2 would be observed only with the sterilization of a pure culture in one physiological form, under ideal sterilization conditions. The value of *k* is not only species dependent, but dependent on the physiological form of the cell; for example, the endospores of the genus *Bacillus* are far more

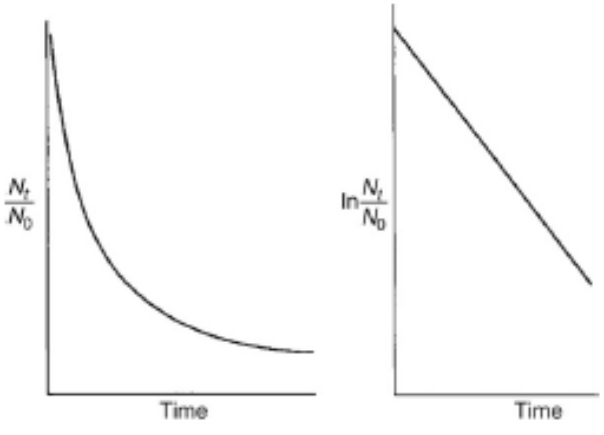


FIGURE 5.2 Plots of the Proportion of Survivors and the Natural Logarithm of the Proportion of Survivors in a Population of Microorganisms Subjected to a Lethal Temperature Over a Time Period

heat resistant than the vegetative cells. Richards (1968) produced a series of graphs illustrating the deviation from theory that may be experienced in practice. Fig. 5.3a,b and c illustrate the effect of the time of heat treatment on the survival of a population of bacterial endospores. The deviation from an immediate exponential decline in viable spore number is due to the heat activation of the spores, that is the induction of spore germination by the heat and moisture of the initial period of the sterilization process. In Fig. 5.3a, the activation of spores is significantly more than their destruction during the early stages of the process and, therefore, viable numbers increase

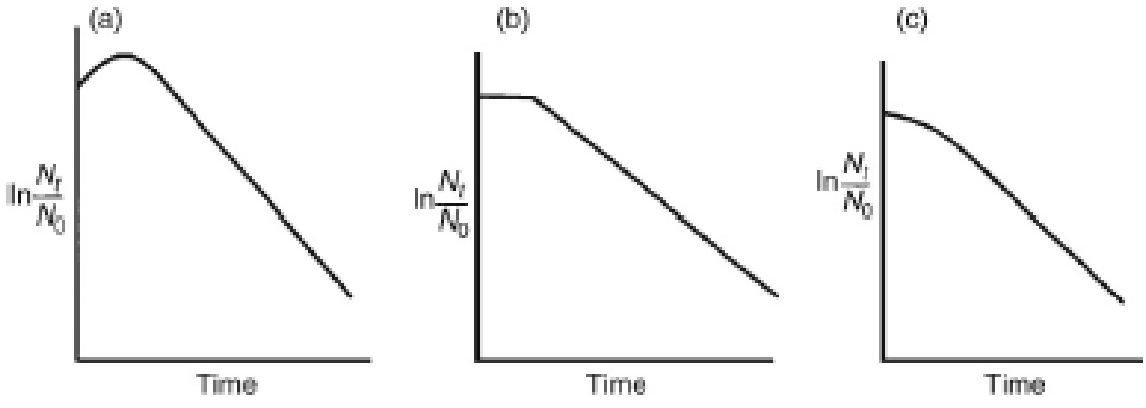


FIGURE 5.3 Plots of the Behavior of a Population of Bacterial Spores Subjected to a Lethal temperature Over a Time Period

(a) Initial population increase resulting from the heat activation of spores in the early stages of a sterilization process (Richards, 1968). (b) An initial stationary period observed during a sterilization treatment due to the death of spores being completely compensated by the heat activation of spores (Richards, 1968). (c) Initial population decline at a sub-maximum rate during a sterilization treatment due to the death of spores being compensated by the heat activation of spores (Richards, 1968).

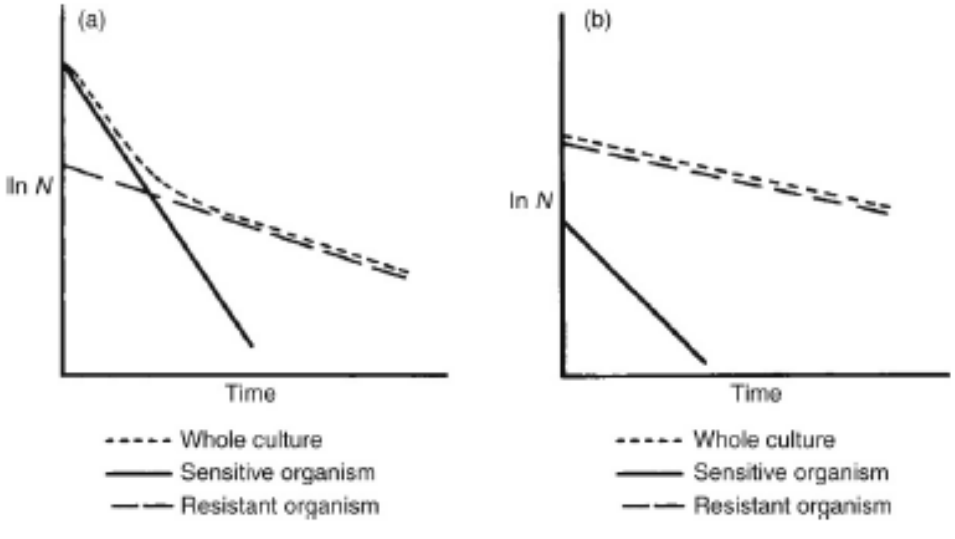


FIGURE 5.4 Plots of the Behavior of Mixed Microbial Cultures Subjected to a Lethal Temperature Over a Time Period

(a) The effect of a sterilization treatment on a mixed culture consisting of a high proportion of a very sensitive organism (Richards, 1968). (b) The effect of a sterilization treatment on a mixed culture consisting of a high proportion of a relatively resistant organism (Richards, 1968). before the observation of exponential decline. In Fig. 5.3b activation is balanced by spore death and in Fig. 5.3c activation is less than spore death. Fig. 5.4a and b illustrate typical results of the sterilization of mixed cultures containing two species with different heat sensitivities. In Fig. 5.4a the population consists mainly of the less-resistant type where the initial decline is due principally to the destruction of the less-resistant cell population and the later, less rapid decline, is due principally to the destruction of the more resistant cell population. Fig. 5.4b represents the reverse situation where the more resistant type predominates and its presence disguises the decrease in the number of the less resistant type. As with any first-order reaction, the reaction rate increases with increase in temperature due to an increase in the reaction rate constant, which, in the case of the destruction of microorganisms, is the specific death rate (k). Thus, k is a true constant only under constant temperature conditions. The relationship between temperature and the reaction rate constant was demonstrated by Arrhenius and may be represented by the equation:

$$\frac{d \ln k}{dT} = \frac{E}{RT^2}$$

! is the gas constan (5.4)

where E is the activation energy, R is the gas constant, T is the absolute temperature.
On integration Eq. (5.4) gives:

$$k = Ae^{-E/RT}$$

where A is the Arrhenius constant.

On taking natural logarithms, Eq. (5.5) becomes:

$$\ln k = \ln A - \frac{E}{RT} \quad (5.6)$$

From Eq. (5.6), it may be seen that a plot of ln k against the reciprocal of the absolute temperature will give a straight line. Such a plot is termed an Arrhenius plot and enables the calculation of the

activation energy and the prediction of the reaction rate for any temperature. By combining Eqs. (5.3) and (5.5) together, the following expression may be derived for the heat sterilization of a pure culture at a constant temperature:

$$\ln N_0/N_t = A \cdot t \cdot e^{-(E/RT)}. \tag{5.7}$$

Deindoerfer and Humphrey (1959) used the term ln *N0/Nt* as a design criterion for sterilization, which has been variously called the Del factor, Nabla factor, and sterilization criterion represented by the term ∇. Thus, the Del factor is a measure of the fractional reduction in viable organism count produced by a certain heat and time regime. Therefore:

$$\nabla = \ln(N_0/N_t)$$

but

$$\ln(N_0/N_t) = kt$$

and

$$kt = A \cdot t \cdot e^{-(E/RT)}$$

thus

$$\nabla = A \cdot t \cdot e^{-(E/RT)}. \tag{5.8}$$

On taking natural logarithms and rearranging, Eq. (5.8) becomes:

$$\ln t = \frac{E}{RT} + \ln\left(\frac{\nabla}{A}\right) \tag{5.9}$$

Thus, a plot of the natural logarithm of the time required to achieve a certain ∇ value against the reciprocal of the absolute temperature will yield a straight line, the slope of which is dependent on the activation energy, as shown in Fig. 5.5. From Fig. 5.5 it is clear that the same degree of sterilization (∇) may be obtained over a wide range of time and temperature regimes; that is, the same degree of sterilization may result from treatment at a high temperature for a short time as from a low temperature for a long time.

This kinetic description of bacterial death enables the design of procedures (giving certain ∇ factors) for the sterilization of fermentation broths. By choosing a value for *Nt*, procedures may be designed having a certain probability of achieving sterility, based upon the degree of risk that is considered acceptable.

According to Deindoerfer and Humphrey (1959), Richards (1968), Banks (1979), and Corbett (1985) a risk factor

of one batch in a thousand being contaminated is frequently used in the fermentation industry—that is, the final microbial count in the medium after sterilization should be 10–3 viable cells. However, to apply these kinetics, it is necessary to know the thermal death characteristics of all the taxa contaminating the fermenter and unsterile medium. This is an impossibility and, therefore, the assumption may be made that the only microbial contaminants present are spores of *Geobacillus stearothermophilus*—that is, one of the most heat-resistant microbial types known. *G. stearothermophilus* was previously included in the genus *Bacillus* as *Bacillus stearothermophilus* until its reclassification in 2001 (Nazina et al., 2001). Thus, by adopting *G. stearothermophilus* as the design organism a considerable safety factor should be built into the calculations. It should be remembered that *G. stearothermophilus* is not always adopted as the design organism. If the most heat-resistant organism contaminating the medium ingredients is known, then it may be advantageous to base the sterilization process on this organism. Deindoerfer and Humphrey (1959) determined the thermal death characteristics of *G. stearothermophilus* spores as:

Activation energy 283.3 $kJmol^{-1}$

Arrhenius constant 1 10 $second^{-1}$

However, it should be remembered that these kinetic values will vary according to the medium in which the spores are suspended, and this is particularly relevant

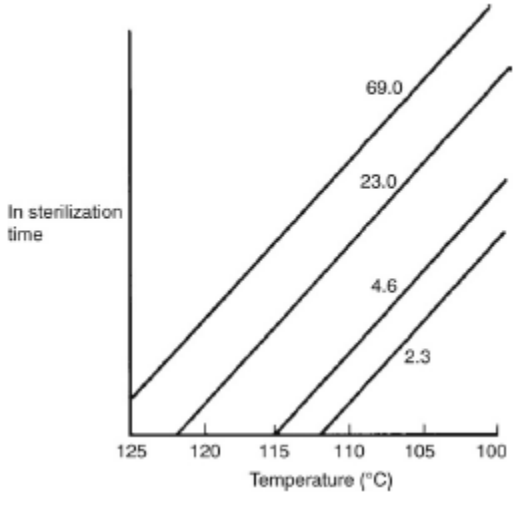


FIGURE 5.5 The Effect of Sterilization and Temperature on the Del Factor Achieved in the Process

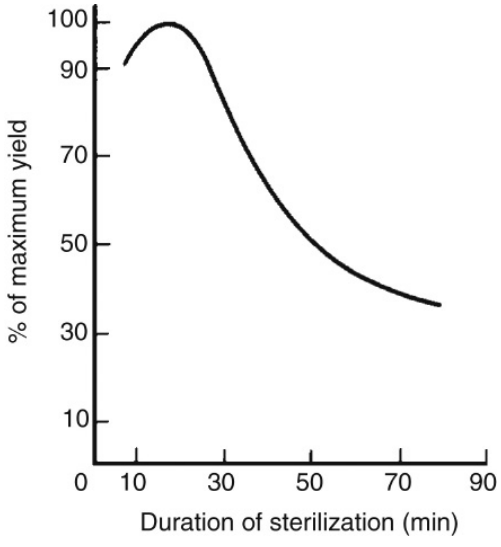


FIGURE 5.6 The Effect of the Time of Sterilization on the Yield of a Subsequent Fermentation

when considering the sterilization of fats and oils (which are common fermentation substrates) where the relative humidity may be quite low. Bader, Boekeloo, Graham, and Cagle (1984) demonstrated that spores of *Bacillus macerans* suspended in oil were ten times more resistant to sterilization if they were dry than if they were wet. A regime of time and temperature may now be determined to achieve the desired Del factor. However, a fermentation medium is not an inert mixture of components, and deleterious reactions may occur in the medium during the sterilization process, resulting in a loss of nutritive quality. Thus, the choice of regime is dictated by the requirement to achieve the desired reduction in microbial content with the least detrimental effect on the medium. Fig. 5.6 illustrates the deleterious effect of increasing medium sterilization time on the yield of product of subsequent fermentations. The initial rise in yield is due to some components of the medium being made more available to the process microorganism by the "cooking effect" of a brief sterilization period (Richards, 1966).

Two types of reaction contribute to the loss of nutrient quality during sterilization:

1. *Interactions between nutrient components of the medium.* A common occurrence during sterilization is the Maillard-type browning reaction that results in discoloration of the medium as well as loss of nutrient quality and the accumulation of growth-inhibitory compounds (Helou et al., 2014). These highly complex reactions normally occur between carbonyl groups, usually from reducing sugars, with the amino groups of amino acids and proteins. An example of the effect of

sterilization time on the availability of glucose in a corn-steep liquor medium is shown in Table 5.1 (Corbett, 1985). Problems of this type can be resolved by sterilizing the sugar separately from the rest of the medium and recombining the two after cooling—as is done at a laboratory scale (when sterilizing medium using an autoclave) and effectively in fed-batch fermentations. Marshall, Lilly, Gbewonyo, and Buckland (1995) measured the interactions between glucose and proteins caused by sterilization using changes in the absorption spectra and pH of a fermentation medium pre- and poststerilization. They demonstrated that a recombinant *E. coli* strain producing acidic fibroblast growth factor performed significantly better when glucose and protein were sterilized separately and the better performance correlated with decreased interaction between the two ingredients.

2. *Degradation of heat labile components.* Certain vitamins, amino acids, and proteins may be degraded during a steam sterilization regime.

Advantages of continuous sterilization over batch sterilization:

1. Superior maintenance of medium quality.

2. Ability to sterilize medium components separately.

3. Superior energy efficiency, consuming 60–80% less steam and cooling water.

4. Ease of scale-up—discussed later.

5. Easier automatic control.

6. The reduction of surge capacity for steam.

7. The reduction of sterilization cycle time and hence the reduction in fermenter turn around time, thus increasing productivity.

8. Under certain circumstances, the reduction of fermenter corrosion.

9. Enables the use of a lower capacity agitator in the fermenter giving economies in both capital and running costs.

Advantages of batch sterilization over continuous sterilization

1. Lower capital equipment costs.

2. Lower risk of contamination—continuous processes require the aseptic transfer of the sterile broth to the sterile vessel.

3. Easier manual control.

4. Easier to use with media containing a high proportion of solid matter. The early continuous sterilizers were constructed as plate heat exchangers and these were unsuitable on two accounts:

1. Failure of the gaskets between the plates resulted in the mixing of sterile and unsterile streams.

2. Particulate components in the media would block the heat exchangers. However, modern continuous sterilizers use double spiral heat exchangers in which the two streams are separated by a continuous steel division. Also, the spiral exchangers are far less susceptible to blockage. However, a major limitation to the adoption of continuous sterilization was the precision of control necessary for its success. This precision has been achieved with the development of sophisticated computerized monitoring and control systems resulting in continuous sterilization being very widely used and it is now the method of choice. However, batch sterilization is still used in many fermentation plants and, thus, it will be considered here before continuous sterilization is discussed in detail.

DESIGN OF BATCH STERILIZATION PROCESSES

Although a batch sterilization process is less successful in avoiding the destruction of nutrients than a continuous one, the objective in designing a batch process is still to achieve the required probability of obtaining sterility with the minimum loss of nutritive quality. The highest temperature, which appears to be feasible for batch sterilization is 121°C so the procedure should be designed such that exposure of the medium to this temperature is kept to a minimum. This is achieved by taking into account the contribution made to the sterilization by the heating and cooling periods of the batch treatment. Deindoerfer and Humphrey (1959) presented a method to assess the contribution made by the heating and cooling periods. The following information must be available for the design of a batch sterilization process:

1. A profile of the increase and decrease in the temperature of the fermentation medium during the heating and cooling periods of the sterilization cycle.

2. The number of microorganisms originally present in the medium.

3. The thermal death characteristics of the "design" organism. As explained earlier this may be *G. stearothermophilus* or an alternative organism relevant to the particular fermentation.

Knowing the original number of organisms present in the fermenter and the risk of sterilization failure considered acceptable, the required Del factor may be calculated. A frequently adopted risk of sterilization failure is 1 in 1000. This value should not be confused with the acceptable rate of contamination previously quoted as 1 in 100 as contamination results from failures of processes other than medium sterilization, including air sterilization, cooling equipment leaks, and aseptic operation failure. A sterilization failure rate of 1 in 1000 is commensurate with Nt being equal to

10–3 of a viable cell. It is worth reinforcing at this stage that we are considering the absolute total number of organisms present in the volume of medium being sterilized and *not* the concentration. If a specific case is considered where the unsterile broth was shown to contain

$$\nabla = \ln(10^{11}/10^{-3})$$
$$\nabla = \ln 10^{14}$$
$$= 32.2.$$

10^{11} viable organisms, then the Del factor may be calculated, thus: Therefore, the overall Del factor required is 32.2. However, the destruction of cells occur during the heating and cooling of the broth as well as during the period at 121°C, thus, the overall Del factor may be represented as:

$$\nabla_{\text{overall}} = \nabla_{\text{heating}} + \nabla_{\text{holding}} + \nabla_{\text{cooling}}.$$

Knowing the temperature–time profile for the heating and cooling of the broth (prescribed by the characteristics of the available equipment) it is possible to determine the contribution made to the overall Del factor by these periods. Thus, knowing the Del factors contributed by heating and cooling, the holding time may be calculated to give the required overall Del factor.

CALCULATION OF THE DEL FACTOR DURING HEATING AND COOLING

The relationship between Del factor, the temperature and time is given by Eq. (5.8):

$$\nabla = A \cdot t \cdot e^{-(E/RT)}.$$

However, during the heating and cooling periods the temperature is not constant and, therefore, the calculation of ∇ would require the integration of Eq. (5.8) for the time–temperature regime observed. Deindoerfer and Humphrey (1959) produced integrated forms of the equation for a variety of temperature–time profiles, including linear, exponential, and hyperbolic. However, the regime observed in practice is frequently difficult to classify, making the application of these complex equations problematic. Richards (1968) demonstrated the use of a graphical method of integration and this is illustrated in Fig. 5.8. The time axis is divided into a number of equal increments t1, t2, t3, etc., Richards suggesting 30 as a reasonable number. For each increment, the temperature corresponding to the midpoint time is recorded. It may now be approximated that the total Del factor of the heating-up period is equivalent to the sum of the Del factors of the midpoint

temperatures for each time increment. The value of the specific death rate of G. stearothermophilus spores at each midpoint temperature may be deduced from the Arrhenius equation using the

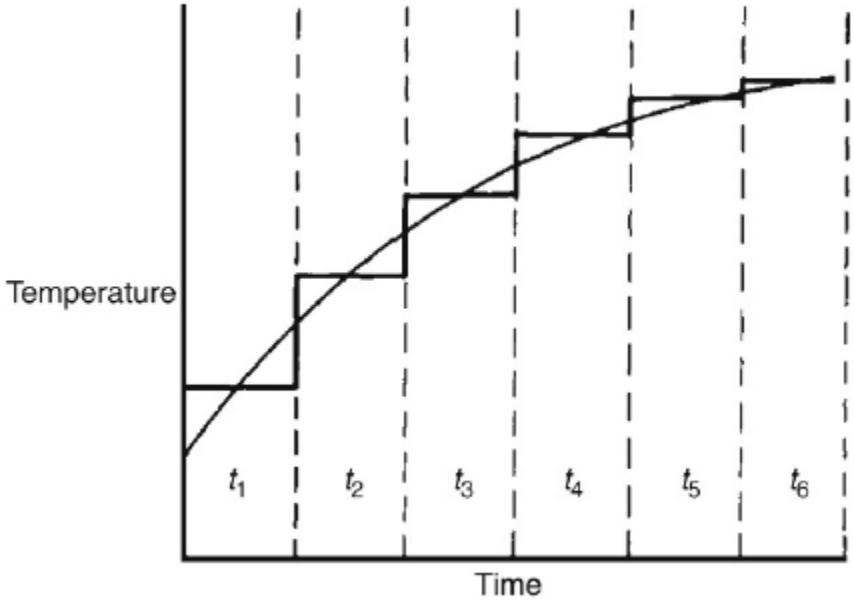


FIGURE 5.8 The Graphical Integration Method Applied to the Increase in Temperature Over a Time Period *t*1, *t*2, etc Represent Equal Time Intervals

thermal death characteristic published by Deindoerfer and Humphrey (1959). The value of the Del factor corresponding to each time increment may then be calculated from the equations:

$$\nabla_1 = k_1 t,$$
$$\nabla_2 = k_2 t,$$
$$\nabla_3 = k_3 t,$$
$$\text{etc.}$$

The sum of the Del factors for all the increments will then equal the Del factor for the heating-up period. The Del factor for the cooling-down period may be calculated in a similar fashion.

METHODS OF BATCH STERILIZATION

The batch sterilization of the medium for a fermentation may be achieved either in the fermentation vessel or in a separate mash cooker. Richards (1966) considered the relative merits of in situ medium sterilization and the use of a special vessel. The major advantages of a separate medium sterilization vessel may be summarized as:

1. One cooker may be used to serve several fermenters and the medium may be sterilized as the fermenters are being cleaned and prepared for the next fermentation, thus saving time between fermentations.

2. The medium may be sterilized in a cooker in a more concentrated form than would be used in the fermentation and then diluted in the fermenter with sterile water prior to inoculation. This would allow the construction of smaller cookers.

3. In some fermentations, the medium is at its most viscous during sterilization and the power requirement for agitation is not alleviated by aeration as it would be during the fermentation proper. Thus, if the requirement for agitation during in situ sterilization were removed, the fermenter could be equipped with a less powerful motor. Obviously, the sterilization kettle would have to be equipped with a powerful motor, but this would provide sterile medium for several fermenters.

4. The fermenter would be spared the corrosion that may occur with medium at high temperature.

The major disadvantages of a separate medium sterilization vessel may be summarized as:

1. The cost of constructing a batch medium sterilizer is much the same as that for the fermenter.

2. If a cooker serves a large number of fermenters complex pipework would be necessary to transport the sterile medium, with the inherent dangers of contamination.

3. Mechanical failure in a cooker supplying medium to several fermenters would render all the fermenters temporarily redundant. The provision of contingency equipment may be prohibitively costly.

Overall, the pressure to decrease the "down time" between fermentations has tended to outweigh the perceived disadvantages of using separate sterilization vessels. Thus, sterilization in dedicated vessels is the method of choice for batch sterilization. However, as pointed out by Corbett (1985), the fact that separate batch sterilizers are used, lends further weight to the argument that continuous sterilization should be adopted in preference to batch. The capital cost of a separate batch sterilizer is similar to that of a continuous one and the problems of transfer of sterile media are then the same for both batch and continuous sterilization. Thus, two of the major objections to continuous systems (capital cost and aseptic transfer) may be considered as no longer relevant.

DESIGN OF CONTINUOUS STERILIZATION PROCESSES

The design of continuous sterilization cycles may be approached in exactly the same way as for batch sterilization systems. The continuous system includes a time period during which the medium is heated to the sterilization temperature, a holding time at the desired temperature, and a cooling period to restore the medium to the fermentation temperature. The temperature of the medium is elevated in a continuous heat exchanger and is then maintained in an insulated serpentine holding coil for the holding period. The length of the holding period is dictated by the

length of the coil and the flow rate of the medium. The hot medium is then cooled to the fermentation temperature using two sequential heat exchangers—the first utilizing the incoming medium as the cooling source (thus conserving heat by heating-up the incoming medium) and the second using cooling water. As discussed earlier, the major advantage of the continuous process is that a much higher temperature may be utilized, thus reducing the holding time and reducing the degree of nutrient degradation. The required Del factor may be achieved by the combination of temperature and holding time that gives an acceptably small degree of nutrient decay. Richards (1968) quoted the following example to illustrate the range of temperature-time regimes that may be employed to achieve the same probability of obtaining sterility. The Del factor used by Richards for the example sterilization was 45.7 and the following temperature time regimes were calculated such that they all gave the same Del factor value of 45.7:

Furthermore, because a continuous process involves treating small increments of medium, the heating-up and cooling-down periods are very small compared with those in a batch system. There are two types of continuous sterilizer that may be used for the treatment of fermentation media: the indirect heat exchanger and the direct heat exchanger (steam injector). The most suitable indirect heat exchangers are of the double-spiral type, which consists of two sheets of high-grade stainless steel, which have been curved around a central axis to form a double spiral, as shown in Fig. 5.11. The ends of the spiral are sealed by covers. A full-scale example is shown in Fig. 5.12. To achieve sterilization

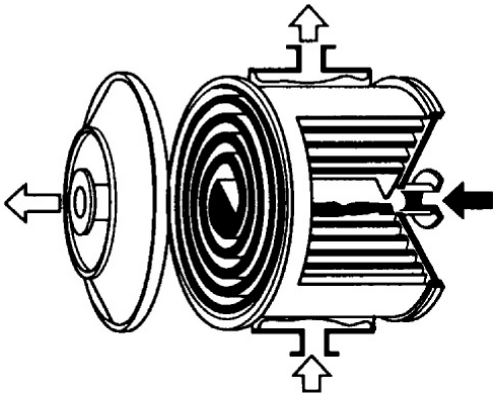


FIGURE 5.11 A Schematic Representation of a Spiral Heat Exchanger

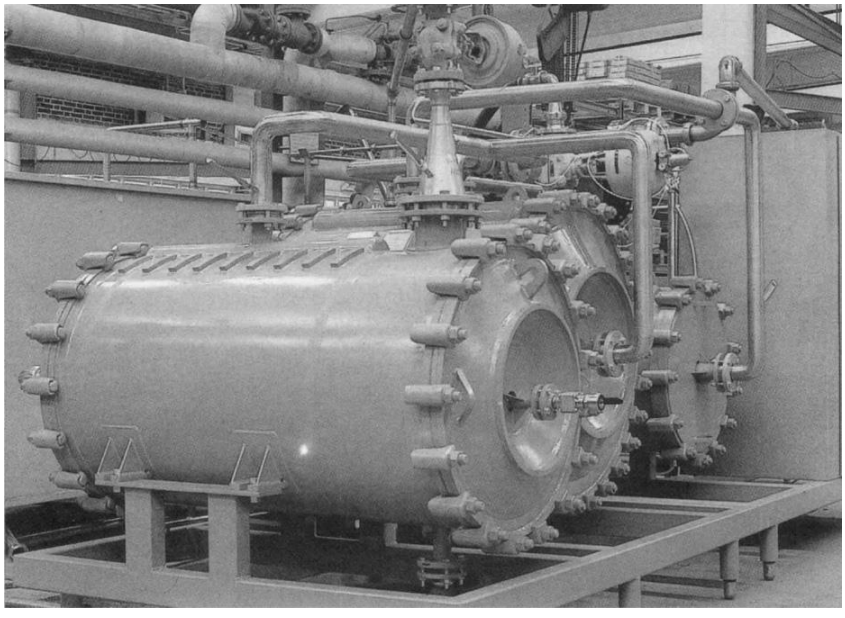


FIGURE 5.12 Industrial Scale Spiral Heat Exchanger

temperatures steam is passed through one spiral from the center of the exchanger and medium through the other spiral from the outer rim of the exchanger, in countercurrent streams. Spiral heat exchangers are also used to cool the medium after passing through the holding coil. Incoming unsterile medium is used as the cooling agent in the first cooler so that the incoming medium is partially heated before it reaches the sterilizer and, thus, heat is conserved.

The major advantages of the spiral heat exchanger are:

1. The two streams of medium and cooling liquid, or medium and steam, are separated by a continuous stainless steel barrier with gasket seals being confined to the joints with the end plates. This makes cross contamination between the two streams unlikely.

2. The spiral route traversed by the medium allows sufficient clearances to be incorporated for the system to cope with suspended solids. The exchanger tends to be self-cleaning which reduces the risk of sedimentation, fouling, and "burning-on." Indirect plate heat exchangers consist of alternating plates through which the countercurrent streams are circulated. Gaskets separate the plates and failure of these gaskets can cause cross-contamination between the two streams. Also, the clearances between the plates are such that suspended solids in the medium may block the exchanger and, thus, the system is only useful in sterilizing completely soluble media. However, the plate exchanger is more adaptable than the spiral system in that extra plates may be added to increase its capacity. The continuous steam injector injects steam directly into the unsterile broth. The advantages and disadvantages of the system have been summarized by Banks (1979):

1. Very short (almost instantaneous) heating up times.

2. It may be used for media containing suspended solids.

3. Low capital cost.

4. Easy cleaning and maintenance.

5. High steam utilization efficiency.

However, the disadvantages are:

1. Foaming may occur during heating.

2. Direct contact of the medium with steam requires that allowance be made for condense dilution and requires "clean" steam, free from anticorrosion additives. In some cases the injection system is combined with flash cooling, where the sterilized medium is cooled by passing it through an expansion valve into a vacuum chamber. Cooling then occurs virtually instantly. A flow chart of a continuous sterilization system using direct steam injection is shown in Fig. 5.13. In some cases a combination of direct and indirect heat exchangers may be used (Svensson, 1988). This is especially true for starch-containing broths when steam injection is used for the preheating step. By raising the temperature virtually instantaneously, the critical gelatinization temperature of the starch is passed through very quickly and the increase in viscosity normally associated with heated starch colloids can be reduced.

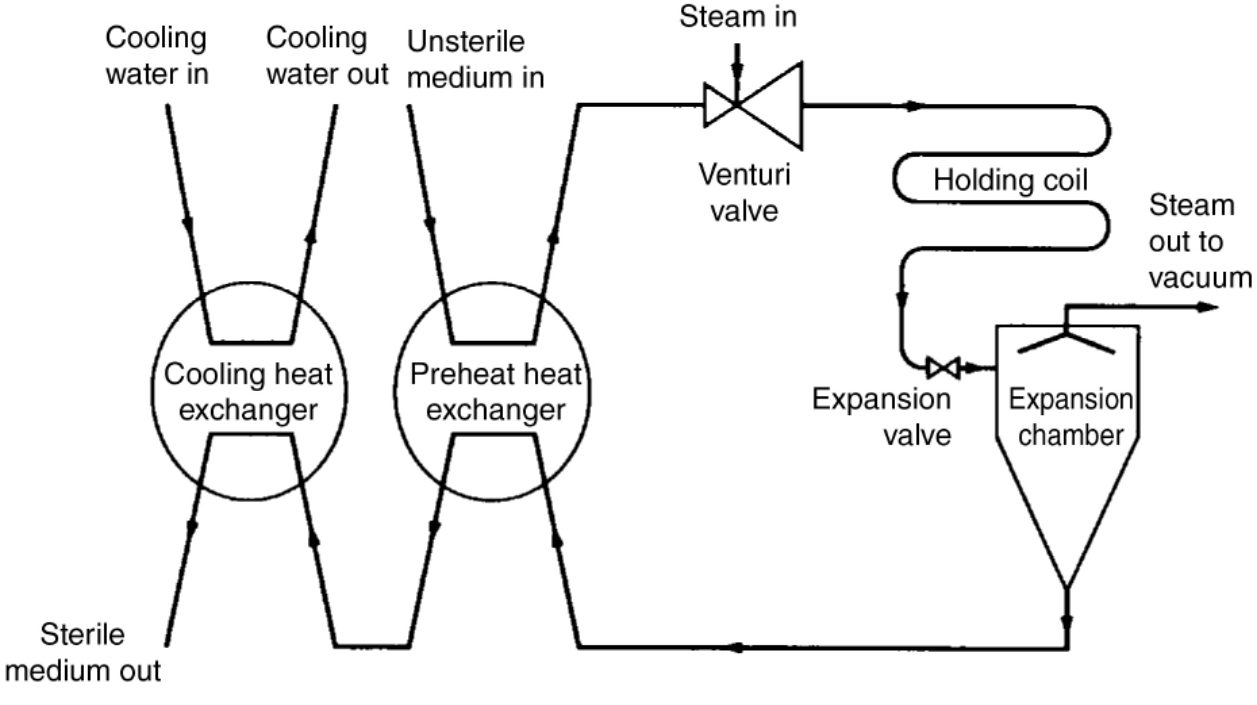


FIGURE 5.13 Flow Diagram of a Typical Continuous Injector-Flash Cooler Sterilizer

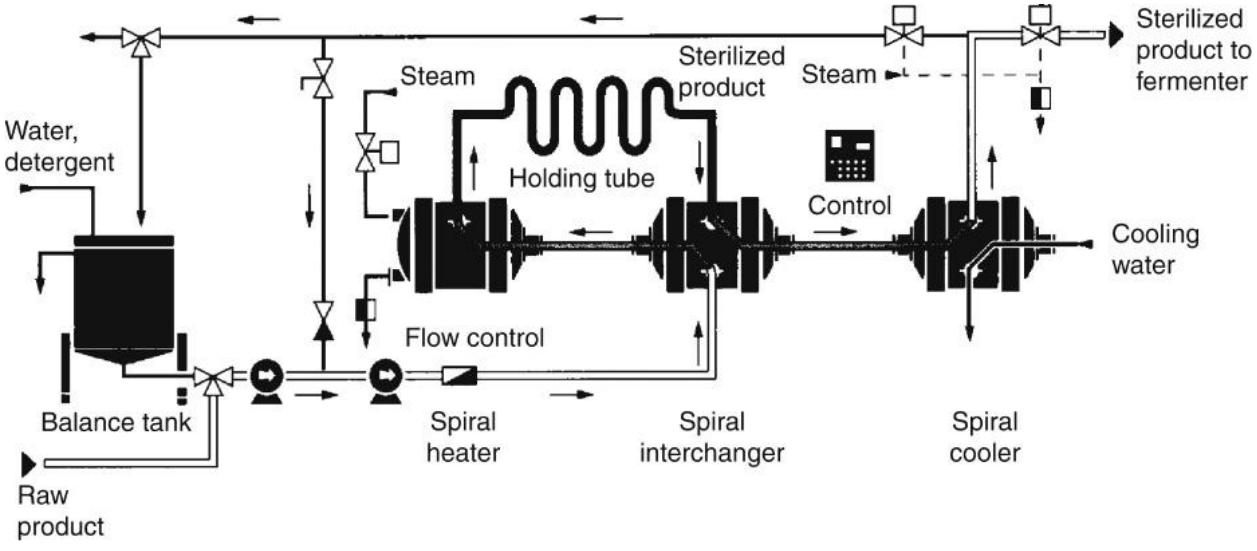


FIGURE 5.14 Flow Diagram of a Typical Continuous Sterilization System Employing Spiral Heat Exchangers

The most widely used continuous sterilization system is that based on the spiral heat exchangers and a typical layout is shown in Fig. 5.14. Junker et al. (2006) described the replacement of a pilot-plant direct-steam injection process with one based on indirect spiral heat exchangers, thus avoiding the problems of the medium quality being affected by fluctuations in the plant steam quality—particularly the presence of steam anticorrosion additives. The key features of this plant were:

1. A number of feed tanks from 2,000 to 19,000 dm3 that enabled both a range of medium volumes to be processed and different medium ingredients to be sterilized sequentially and separately.

2. A recycle tank (also referred to as a circulation or surge tank) and recycle facility so that water could be circulated through the system during cleaning and sterilization of the plant.

3. A spiral heat exchanger to raise the temperature of the medium to the sterilization temperature.

4. A "retention loop" to hold the medium at the sterilization temperature for the required time.

5. A "recuperator" spiral heat exchanger to cool the sterilized medium leaving the retention loop against incoming cold, unsterile medium, thus minimizing heat wastage.

6. A cooling spiral heat exchanger supplied with chilled water to cool the sterilized medium leaving the recuperator exchanger to the process temperature.

7. An additional cooling spiral heat exchanger used when the system was used to produce sterile water.

8. A switching station to direct the product stream from the final coolers:

a. to a distribution system to the fermenters (if medium is sterile),

b. to the recycle tank for recirculation through the system during sterilization of the system, and **c.** to the sewer during cleaning of the system or should a malfunction occur. The Del factor to be achieved in a continuous sterilization process has to be increased with an increase in scale, and this is calculated exactly as described in the consideration of the scale up of batch regimes. Thus, if the volume to be sterilized is increased from 1000 to 10,000 dm3 and the risk of failure is to remain at 1 in 1000 then the Del factor must be increased from 34.5 to 36.8. However, the advantage of the continuous process is that temperature may be used as a variable in scaling up a continuous process so that the increased ∇ factor may be achieved while maintaining the nutrient quality constant. A further advantage of the continuous process is that the distribution system can be devised such that a sterile medium stream can be diverted for use on a small scale. Thus, laboratory or small fermenter scale models of the process can be conducted using medium that has been exposed to exactly the same sterilization regime as the production scale. Deindoerfer and Humphrey (1961) discussed

STERILIZATION BY FILTRATION

The range of operations associated with a fermentation process that may involve sterilization by filtration are illustrated in Fig. 5.18 from which it may be seen that both liquid and gas filtration are utilized. The removal of suspended solids from both gas and liquids in these processes may then be described by the following mechanisms:

1. Inertial impaction.

2. Diffusion.

3. Electrostatic attraction.

4. Interception.

1. *Inertial impaction.* Suspended particles in a fluid stream have momentum. The fluid in which the particles are suspended will flow through the filter by the route of least resistance. However, the particles because of their momentum tend to travel in straight lines and may therefore become impacted upon the fibers, where they may then remain. Inertial impaction is more significant in the filtration of gases than in the filtration of liquids.

2. *Diffusion.* Extremely small particles suspended in a fluid are subject to Brownian motion that is random movement due to collisions with fluid molecules. Thus, such small particles tend to deviate from the fluid flow pattern and may become impacted upon the filter fibers. Diffusion is more significant in the filtration of gases than in the filtration of liquids.

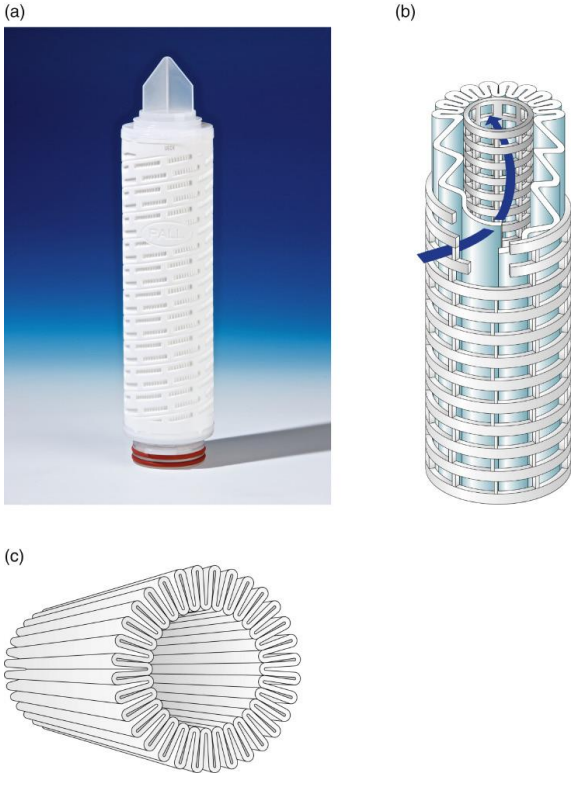
3. *Electrostatic attraction.* Charged particles may be attracted by opposite charges on the surface of the filtration medium.

4. *Interception.* The fibers comprising a filter are assembled to define openings of various sizes. Particles that are larger than the filter pores are removed by direct interception. However, a significant number of particles that are smaller than the filter pores are also retained by interception. This may occur by several mechanisms—more than one particle may arrive at a pore simultaneously, an irregularly shaped particle may bridge a pore, once a particle has been trapped by a mechanism other than interception the pore may be partially occluded enabling the entrapment of smaller particles. Interception is equally important mechanism in the filtration of gases and liquids.

Filters have been classified into two types—those in which the pores in the filter are smaller than the particles that are to be removed and those in which the pores are larger than the particles that are to be removed. The former type may be regarded as an absolute filter, so that filters of this type

(provided they are not physically damaged) are claimed to be 100% efficient in removing microorganisms. Filters of the latter type are frequently referred to as depth filters, originally composed of felts, woven yarns, and loosely packed fiberglass in packed towers and were widely used for air filtration. However, modern depth filters tend to be manufactured as immobilized fibers (microglass and synthetic polymers) to form a sheet or cartridge that are then accommodated in specialized housings. These systems then avoid the previous problem of packed towers in which an increase in the pressure on the filter may result in movement of the material, producing larger channels through the filter, resulting in the loss of integrity. The terms absolute and depth can be misleading as they imply that absolute filtration only occurs at the surface of the filter, whereas absolute filters also have depth and thus filtration occurs within the filter as well as at the surface.Terms that bear more relationship to the construction of filters are "nonfixed pore filters" (corresponding with depth filters) and "fixed pore filters" (corresponding with absolute filters). Nonfixed pore filters rely on the removal of particles by inertial impaction, diffusion, and electrostatic attraction rather than interception. The packing material contains innumerable tortuous routes through the filter but removal is a statistical phenomenon and, thus, sterility of the product is predicted in terms of the probability of failure (similar to the situation for steam sterilization). Thus, in theory, the removal of microorganisms by a fibrous filter cannot be absolute as there is always the possibility of an organism passing through the filter, regardless of the filter's depth. However, modern nonfixed pore filters can achieve an exceptionally small probability of failure, 10^{-20} or less, such that they may achieve performances equivalent to a fixed pore filter (Wikol et al., 2008) and may be described by a manufacturer as "rated as absolute"—another reason to avoid describing filters as "depth" or "absolute." It is possible that increased pressure applied to a nonfixed pore filter may result in the displacement of previously trapped particles. Fixed pore filters are constructed so that the filtration medium will not be distorted during operation so that the flow patterns through the filter will not change due to disruption of the material. Pore size is controlled during manufacture so that an absolute rating can be quoted for the filter, that is, the removal of particles above a certain size can be guaranteed. Thus, interception is the major mechanism by which particles are removed. Because fixed pore filters have depth, they are also capable of removing particles that are smaller than the pores by the mechanisms of inertial impaction, diffusion, and attraction and these mechanisms do play significant roles in the filtration of gases. Fixed pore filters are superior for most purposes such that they have absolute ratings, are less susceptible to changes in pressure, and are less likely to release trapped particles.

The major disadvantage associated with fixed pore filters was the resistance to flow they presented and, hence, the large pressure drop across the filters which represents a major operating cost. Filtration companies have developed both fixed pore and nonfixed pore filters based on filter sheets that are pleated and incorporated into a cartridge device in which the filter membrane is supported, and protected from physical damage, by a plastic skeleton. These devices incorporate a large membrane surface area and have minimized the problems associated with early filtration systems. The large surface area reduces pressure-drop across the membrane of fixed pore filters and in nonfixed pore filters the material is immobilized in the cartridge, rather than being loosely packed in a tower, thus preventing its movement during filtration. The cartridges are then accommodated in stainless steel housings and scale-up can then be achieved conveniently by increasing the number of cartridges. The structure of a filter cartridge



(a) A filter cartridge. (b) A schematic diagram showing the pleated membrane and supporting structure of a cartridge. (c) A schematic diagram showing the pleated membrane and its pleated membrane is shown in Fig. 5.19 and a range of cartridge types in Fig. 5.20a. Examples of stainless steel housings can be seen in Fig. 5.21. It is important to realize that the filters, themselves, must be sterile and this is achieved in large-scale microbial processes by steam sterilization before and after operation (see Chapter 7). Thus, the materials must be stable at high temperatures and the steam must be free of particulate

matter because the filter modules are particularly vulnerable to damage at high temperatures, achieved by filtering the steam

(b)

FIGURE 5.20

A selection of (a) filter cartridges to be used with stainless steel housings, (b) presterilized plastic capsules containing the filter cartridge.VC itself through stainless steel mesh filters rated at 1 μm. However, filters for both air and liquid filtration are also available as disposable (single-use) capsules in which the pleated filter membrane is welded into a plastic housing with accompanying connections and tubing. Such systems are provided presterilized by gamma irradiation and examples are shown in Fig. 5.20b. The availability of disposable presterilized mixing

FIGURE 5.21 Examples of Stainless-Steel Cartridge

Filter Housings and holding vessels, along with associated pumps and disposable filters, has enabled the adoption of entire disposable trains from medium preparation through to addition of the sterile medium to a presterilized single-use fermenter, supplied with sterile air via a disposable filter assembly. These single-use systems are predominantly used in animal cell fementations but have also found popularity in small-scale microbial fermentations utilizing disposable reactors, for example, heterologous protein manufacture, or for the production of inocula for large-scale traditional processes.

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STOICHIOMETRY OF MICROBIAL GROWTH AND PRODUCT FORMATION

Cell growth and product formation are complex processes reflecting the overall kinetics and stoichiometry of the thousands of intracellular reactions that can be observed within a cell. For many process calculations, we wish to compare potential substrates in terms of cell mass yield, or product yield, or evolution of heat. Also, we may need to know how close the system is operating to its thermodynamic limits. (That is, is product yield constrained by kinetic or thermodynamic considerations?) If a system is close to its thermodynamic limit, it would be unwise to try to improve production through mutation or genetic engineering.

Although the cell is complex, the stoichiometry of conversion of substrates into products and cellular materials is often represented by a simple pseudochemical equation. In this chapter, we discuss how these equations can be written and how useful estimates of key yield coefficients can be made.

7.1. COEFFICIENTS FOR ATP CONSUMPTION AND OXYGEN

Tthe definitions of yield and maintenance coefficients, and we learned how to estimate their values using chemostat culture. In particular, we discussed the overall growth yield coefficient $Y^M_{X/S}$, which is the maximum yield of cell mass per unit mass of substrate consumed when no maintenance is considered.

Two other yield and maintenance coefficients of importance are related to adenosine triphosphate (ATP) consumption and oxygen. The ATP yield coefficient, $Y_{X/ATP}$, represents the amount of biomass synthesized per mole of ATP generated. Surprisingly, it has been observed that for many substrates and organisms, $Y^M_{X/ATP}$ is nearly constant at 10 to 11 g dry weight/mol ATP for heterotrophic growth under anaerobic conditions. The ATP yield for many autotrophic organisms (recall that autotrophic organisms fix CO_2) is approximately $Y^M_{X/ATP} \approx 6.5$ g/mol ATP. Under aerobic conditions, the values for $Y^M_{X/ATP}$ are usually greater than 10.5 (see Table 7.1).

Organism	Growth-Limiting Factor	m_{ATP} (mol ATP/g-cells-h)	$Y^M_{X/ATP}$ (g cells/mol ATP)
Lactobacillus casei	Glucose	1.5	24.3
Enterobacter aerogenes	Glucose[a]	6.8	14.0
	Glucose[b]	2.3	17.6
	Tryptophan	38.7	25.4
	Citrate	2.2	9.0
Escherichia coli	Glucose	18.9	10.3
		6.9	8.5
Saccharomyces cerevisiae	Glucose	0.5	11.0
		0.25	13.0
Saccharomyces cerevisiae (petite)	Glucose	0.7	11.3
Candida parapsilosis	Glucose	0.2	12.5
Clostridium acetobutylicum	Glucose	—	23.8
Streptococcus cremoris	Lactose[c]	2.3	12.6

[a] Minimal medium.
[b] Complex medium.
[c] In the presence of a high extracellular lactate concentration.
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TABLE 7.1. Growth Parameters of Organisms Growing Anaerobically in a Chemostat

Table 7.2 shows calculated ATP yields (maximum theoretical values) for a variety of media. A maintenance coefficient can also be estimated using an equation analogous to the one we developed for substrate yield coefficient in a chemostat:

Growth Medium	$Y_{X/ATP}$ (g cells/mol ATP)
Glucose + amino acids + nucleic acids	31.9
Glucose + inorganic salts	28.8
Pyruvate + amino acids + nucleic acids	21
Pyruvate + inorganic salts	13.5
CO_2 + inorganic salts (autotrophic growth)	6.5

TABLE 7.2. ATP Yields in Various Growth Media

$$\frac{1}{Y^{AP}_{X/ATP}} = \frac{1}{Y^{M}_{X/ATP}} + \frac{m_{ATP}}{D} \qquad (7.1)$$

Here $Y^{AP}_{X/\mathrm{ATP}}$ is the "apparent" yield of biomass and m_{ATP} is the rate of ATP consumption for maintenance energy.

Similarly, yields based on oxygen consumption can be defined and calculated as follows:

$$\frac{1}{Y^{AP}_{X/\mathrm{O_2}}} = \frac{1}{Y^{M}_{X/\mathrm{O_2}}} + \frac{m_{\mathrm{O_2}}}{D} \qquad (7.2)$$

As indicated in Chapter 6, Table 6.1, values of $Y_{X/O2}$ can vary from 0.17 to 1.5 g biomass/g O_2, depending on substrate and organism.

Information from some measurements can be usefully combined. A particularly important derived parameter is the respiratory quotient (RQ), which is defined as the moles of CO_2 produced per mole of oxygen consumed. The RQ value provides an indication of metabolic state (e.g., aerobic growth versus ethanol fermentation in baker's yeast) and can be used in process control.

The P/O ratio, which is the ratio of phosphate bonds formed per unit of oxygen consumed (gmol P/g atom O). The P/O ratio indicates the efficiency of conversion of reducing power into high-energy phosphate bonds in the respiratory chain. For eucaryotes, the P/O ratio approaches 3 when glucose is the substrate, whereas it is significantly less than 3 in procaryotes. A closely related parameter is the proton/oxygen ratio (H/O). This ratio is the number of H^+ ions released per mole of oxygen consumed. Electron generation is directly related to proton release. Usually, 4 moles of electrons are generated per mole of oxygen consumed. The generation of electrons results in the expulsion of H^+ that can be used directly to drive the transport of some substrates or to generate ATP.

The complexity of mass and energy balances for cellular growth can be decreased greatly through the recognition that some parameters are nearly the same irrespective of the species or substrate involved. These parameters can be referred to as *regularities*. For example, Table 7.1 shows that $Y^{M}_{X/\mathrm{ATP}} \geq 10.5$ g dry wt/mol ATP under aerobic conditions. Three important regularities (identified first by I. G. Minkevich and V. K. Eroshin) are 26.95 kcal/g equivalent of available electrons transferred to oxygen (coefficient of variation of 4%), 4.291 g equivalent of available electrons per quantity of biomass containing 1 g atom carbon, and 0.462 g carbon in biomass per

gram of dry biomass. It has also been observed that $Y_{X/e^-} = 3.14 \pm 0.11\ \text{g}$ dry wt/g equivalent of electrons. These observed average values of cell composition and yields facilitate estimation of other growth-related parameters.

7.2. STOICHIOMETRIC CALCULATIONS

Conservation laws are absolute and powerful aids in understanding biological reactions and systems. Here we discuss the use of elemental balances and analysis of the energetics of reactions as both constraints on biological systems and as tools to understand what such systems are allowed to do.

7.2.1. Elemental Balances

A material balance on biological reactions can easily be written when the compositions of substrates, products, and cellular material are known. Usually, electron–proton balances are required in addition to elemental balances to determine the stoichiometric coefficients in bioreactions. Accurate determination of the composition of cellular material is a major challenge. Variations in cellular composition with different types of organisms are shown in Table 7.3. A typical cellular composition can be represented as $CH_{1.8}O_{0.5}N_{0.2}$. One mole of biological material is defined as the amount containing 1 gram atom of carbon, such as $CH_\alpha O_\beta N_\delta$.

Microorganism	Limiting Nutrient	μ (h^{-1})	Composition (% by wt)							Empirical Chemical Formula	Formula "Molecular" Weight
			C	H	N	O	P	S	Ash		
Bacteria			53.0	7.3	12.0	19.0			8	$CH_{1.666}N_{0.20}O_{0.27}$	20.7
Bacteria			47.1	7.8	13.7	31.3				$CH_2N_{0.25}O_{0.5}$	25.5
Aerobacter aerogenes			48.7	7.3	13.9	21.1			8.9	$CH_{1.78}N_{0.24}O_{0.33}$	22.5
Klebsiella aerogenes	Glycerol	0.1	50.6	7.3	13.0	29.0				$CH_{1.74}N_{0.22}O_{0.43}$	23.7
K aerogenes	Glycerol	0.85	50.1	7.3	14.0	28.7				$CH_{1.73}N_{0.24}O_{0.43}$	24.0
Yeast			47.0	6.5	7.5	31.0			8	$CH_{1.66}N_{0.13}O_{0.40}$	23.5
Yeast			50.3	7.4	8.8	33.5				$CH_{1.75}N_{0.15}O_{0.5}$	23.9
Yeast			44.7	6.2	8.5	31.2	1.08	0.6		$CH_{1.64}N_{0.16}O_{0.52}P_{0.01}S_{0.005}$	26.9
Candida utilis	Glucose	0.08	50.0	7.6	11.1	31.3				$CH_{1.82}N_{0.19}O_{0.47}$	24.0
C. utilis	Glucose	0.45	46.9	7.2	10.9	35.0				$CH_{1.84}N_{0.2}O_{0.56}$	25.6
C. utilis	Ethanol	0.06	50.3	7.7	11.0	30.8				$CH_{1.82}N_{0.19}O_{0.46}$	23.9
C. utilis	Ethanol	0.43	47.2	7.3	11.0	34.6				$CH_{1.84}N_{0.2}O_{0.55}$	25.5

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TABLE 7.3. Elemental Composition of Microorganisms

Consider the following simplified biological conversion in which no extracellular products other than H_2O and CO_2 are produced:

$$CH_mO_n + a\,O_2 + b\,NH_3 \rightarrow c\,CH_\alpha O_\beta N_\delta + d\,H_2O + e\,CO_2 \qquad (7.3)$$

where CH_mO_n represents 1 mole of carbohydrate, and $CH_\alpha O_\beta N_\delta$ stands for 1 mole of cellular material. Simple elemental balances on C, H, O, and N yield the following equations:

$$\begin{aligned} &\text{C:} \quad 1 = c + e \\ &\text{H:} \quad m + 3b = c\alpha + 2d \\ &\text{O:} \quad n + 2a = c\beta + d + 2e \\ &\text{N:} \quad b = c\delta \end{aligned} \qquad (7.4)$$

The RQ is

$$RQ = \frac{e}{a} \qquad (7.5)$$

Equations 7.4 and 7.5 constitute five equations for five unknowns: *a*, *b*, *c*, *d*, and *e*. With a measured value of RQ, these equations can be solved to determine the stoichiometric coefficients.

7.2.2. Degree of Reduction

In more complex reactions, as in the formation of extracellular products, an additional stoichiometric coefficient is added, requiring more information. Also, elemental balances provide no insight into the energetics of a reaction. Consequently, the concept of *degree of reduction* has been developed and used for proton–electron balances in bioreactions. The degree of reduction, γ, for organic compounds may be defined as the number of equivalents of available electrons per gram atom C. The available electrons are those that would be transferred to oxygen upon oxidation of a compound to CO_2, H_2O, and NH_3. The degrees of reduction for some key elements are C = 4, H = 1, N = –3, O = –2, P = 5, and S = 6. The degree of reduction of any element in a compound is equal to the valence of this element. For example, 4 is the valence of carbon in CO_2, and –3 is the valence of N in NH_3. Degrees of reduction for various organic compounds are given in Table 7.4. The following are examples of how to calculate the degree of reduction for substrates:

Compound	Molecular Formula	Degree of Reduction, γ	Weight, *m* (per carbon mole)
Biomass	$CH_{1.64}N_{0.16}O_{0.52}$	4.17 (NH_3)	24.5
	$P_{0.0054}S_{0.005}$[a]	4.65 (N_2) 5.45 (HNO_3)	
Methane	CH_4	8	16.0
n-Alkane	$C_{15}H_{32}$	6.13	14.1
Methanol	CH_4O	6.0	32.0
Ethanol	C_2H_6O	6.0	23.0
Glycerol	$C_3H_8O_3$	4.67	30.7
Mannitol	$C_6H_{14}O_6$	4.33	30.3
Acetic acid	$C_2H_4O_2$	4.0	30.0
Lactic acid	$C_3H_6O_3$	4.0	30.0
Glucose	$C_6H_{12}O_6$	4.0	30.0
Formaldehyde	CH_2O	4.0	30.0
Gluconic acid	$C_6H_{12}O_7$	3.67	32.7
Succinic acid	$C_4H_6O_4$	3.50	29.5
Citric acid	$C_6H_8O_7$	3.0	33.5
Formic acid	CH_2O_2	2.0	46.0
Oxalic acid	$C_2H_2O_4$	1.0	45.0

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TABLE 7.4. Degree of Reduction and Weight of One Carbon Equivalent of One Mole of Some Substrates and Biomass

$$\text{Methane } (CH_4): \quad 1(4) + 4(1) = 8, \quad \gamma = 8/1 = 8$$

$$\text{Glucose } (C_6H_{12}O_6): \quad 6(4) + 12(1) + 6(-2) = 24, \quad \gamma = 24/6 = 4$$

$$\text{Ethanol } (C_2H_5OH): \quad 2(4) + 6(1) + 1(-2) = 12, \quad \gamma = 12/2 = 6$$

A high degree of reduction indicates a low degree of oxidation. That is, $\gamma_{CH4} > \gamma_{EtOH} > \gamma_{glucose}$.

Consider the aerobic production of a single extracellular product:

$$\underset{\text{substrate}}{CH_mO_n} + aO_2 + bNH_3 \rightarrow \underset{\text{biomass}}{cCH_\alpha O_\beta N_\delta} + \underset{\text{product}}{dCH_xO_yN_z} + eH_2O + fCO_2 \qquad (7.6)$$

Following are the degrees of reduction of substrate, biomass, and product:

$$\gamma_s = 4 + m - 2n \qquad (7.7)$$

$$\gamma_b = 4 + \alpha - 2\beta - 3\delta \qquad (7.8)$$

$$\gamma_p = 4 + x - 2y - 3z \tag{7.9}$$

Note that for CO_2, H_2O, and NH_3 the degree of reduction is zero.

Equation 7.6 can lead to elemental balances on C, H, O, and N; an available electron balance; an energy balance; and a total mass balance. Of the equations, only five will be independent. If all the equations are written, then the extra equations can be used to check the consistency of an experimental data set. Because the amount of water formed or used in such reactions is difficult to determine and water is present in great excess, the hydrogen and oxygen balances are difficult to use. For such a data set, we would typically choose a carbon, a nitrogen, and an available-electron balance:

$$c + d + f = 1 \tag{7.10}$$

$$c\delta + dz = b \tag{7.11}$$

$$c\gamma_b + d\gamma_p = \gamma_s - 4a \tag{7.12}$$

With partial experimental data, it is possible to solve this set of equations. Measurements of RQ and a yield coefficient would, for example, allow the calculation of the remaining coefficients. It should be noted that the coefficient, *c*, is $Y_{X/S}$ (on a molar basis) and *d* is $Y_{P/S}$ (also on a molar basis).

An energy balance for aerobic growth follows:

$$Q_0 c\gamma_b + Q_0 d\gamma_p = Q_0\gamma_s - Q_0 4a \tag{7.13}$$

If Q_0 the heat evolved per equivalent of available electrons transferred to oxygen, is constant, equation 7.13 is *not* independent of equation 7.12. Recall that an observed regularity is 26.95 kcal/g equivalent of available electrons transferred to oxygen, which allows the prediction of heat evolution based on estimates of oxygen consumption.

Equations 7.12 and 7.13 also allow estimates of the fractional allocation of available electrons or energy for an organic substrate. Equation 7.12 can be rewritten as

$$1 = \frac{c\gamma_b}{\gamma_s} + \frac{d\gamma_p}{\gamma_s} + \frac{4a}{\gamma_s} \tag{7.14a}$$

$$1 = \xi_b + \xi_p + \varepsilon \tag{7.14b}$$

where ε is the fraction of available electrons in the organic substrate that is transferred to oxygen, ξ_b is the fraction of available electrons that is incorporated into biomass, and ξ_p is the fraction of available electrons that is incorporated into extracellular products.

Example 7.1.

Assume that experimental measurements for a certain organism have shown that cells can convert two-thirds (wt/wt) of the substrate carbon (alkane or glucose) to biomass.

a. Calculate the stoichiometric coefficients for the following biological reactions:

Hexadecane: $C_{16}H_{34} + a\ O_2 + b\ NH_3 \rightarrow c(C_{4.4}H_{7.3}N_{0.86}O_{1.2}) + d\ H_2O + e\ CO_2$

Glucose: $C_6H_{12}O_6 + a\ O_2 + bNH_3 \rightarrow c(C_{4.4}H_{7.3}N_{0.86}O_{1.2}) + d\ H_2O + e\ CO_2$

b. Calculate the yield coefficients $Y_{X/S}$ (g dw cell/g substrate), Y_{X/O_2} (g dw cell/g O_2) for both reactions. Comment on the differences.

Solution

a. For hexadecane:

Amount of carbon in 1 mole of substrate = 16(12)=192 g

Amount of carbon converted to biomass = 192(2/3)=128 g

Then, 128 = c(4.4)(12);c = 2.24.

$$\text{Amount of carbon converted to } CO_2 = 192 - 128 = 64 \text{ g}$$

$$64 = e(12), \quad e = 5.33$$

The nitrogen balance yields

$$14b = c(0.86)(14)$$

$$b = (2.42)(0.86) = 2.085$$

The hydrogen balance is

$$34(1) + 3b = 7.3c + 2d$$

$$d = 12.43$$

The oxygen balance yields

$$2a(16) = 1.2c(16) + 2e(16) + d(16)$$

$$a = 12.427$$

For glucose:

Amount of carbon in 1 mole of substrate is 72 g

Amount of carbon converted to biomass is 72(2/3) = 48 g

Then, 48 = 4.4*c*(12), and thus *c* = 0.909.

Amount of carbon converted to CO_2 is $72 - 48 = 24$ g.

$$24 = 12e; \text{ and thus } e = 2$$

The nitrogen balance yields

$$14b = 0.86c(14)$$

Consequently, $b = 0.782.$

The hydrogen balance is

$$12 + 3b = 7.3c + 2d$$

resulting in $d = 3.854.$

The oxygen balance yields

$$6(16) + 2(16)a = 1.2(16)c + 2(16)e + 16d$$

$$a = 1.473$$

which allows calculation of *a*.

b. For hexadecane:

$$Y_{X/S} = \frac{2.42(\text{MW})_{\text{biomass}}}{(\text{MW})_{\text{substrate}}}$$

$$Y_{X/S} = \frac{2.42(91.34)}{226} = 0.98 \text{ gdw cells/g substrate}$$

$$Y_{X/O_2} = \frac{2.42(\text{MW})_{\text{biomass}}}{12.43(\text{MW})_{O_2}}$$

$$Y_{X/O_2} = \frac{2.42(91.34)}{(12.43)(32)} = 0.557 \text{ gdw cells/g } O_2$$

For glucose:

$$Y_{X/S} = \frac{(0.909)(91.34)}{180} = 0.461 \text{ gdw cells/g substrate}$$

$$Y_{X/O_2} = \frac{(0.909)(91.34)}{(1.473)(32)} = 1.76 \text{ gdw cells/g } O_2$$

The growth yield on more reduced substrate (hexadecane) is higher than that on partially oxidized substrate (glucose), assuming that two-thirds of all the entering carbon is incorporated in cellular structures. However, the oxygen yield on glucose is higher than that on the hexadecane because glucose is partially oxidized.

7.3. THEORETICAL PREDICTIONS OF YIELD COEFFICIENTS

In aerobic fermentations, the growth yield per mole glucose in oxygen molecules is approximately 3.14 ± 0.11 gdw cells/electron, when ammonia is used as the nitrogen source. The number of available electrons per oxygen molecule (O_2) is four. When the number of oxygen molecules per mole of substrate consumed is known, the growth yield coefficient, $Y_{X/S}$, can easily be calculated. Consider the aerobic catabolism of glucose:

$$C_6H_{12}O_6 + 6\,O_2 \rightarrow 6\,CO_2 + 6\,H_2O$$

The total number of available electrons in 1 mole of glucose is 24. The cellular yield per available electron is $Y_{X/S}$ = 24(3.14) = 76 gdw cells/mol glucose.

The predicted growth yield coefficient is $Y_{X/S}$ = 76/180 = 0.4 gdw cells/g glucose. Most measured values of $Y_{X/S}$ for aerobic growth on glucose are 0.38 to 0.51 g/g

The ATP yield ($Y_{X/ATP}$) in anaerobic fermentations is approximately 10.5 ± 2 gdw cells/mol ATP. In aerobic fermentations, this yield varies between 6 and 29. When the energy yield of a metabolic pathway is known (N moles of ATP produced per gram of substrate consumed), the growth yield $Y_{X/S}$ can be calculated using the following equation:

$Y_{X/S} = Y_{X/ATP}N$

Example 7.2.

Estimate the theoretical growth and product yield coefficients for ethanol fermentation by *S. cerevisiae*, as described by the following overall reaction:

$C_6H_{12}O_6 \rightarrow 2\,C_2H_5OH + 2\,CO_2$

Solution

Since $Y_{X/ATP} \approx$ 10.5 gdw/mol ATP and glucose fermentation to ethanol yields 2 ATP/mol of glucose in yeast, we have the following:

$$Y_{X/S} \approx 10.5 \text{ gdw/mol ATP} \cdot 2 \frac{\text{moles ATP}}{180 \text{ g glucose}}$$

or

$Y_{X/S} \approx 0.117$ gdw/g glucose

For complete conversion of glucose to ethanol by the yeast pathway, the following would be the maximal yield:

$$Y_{P/S} = \frac{2(46)}{180} = 0.51 \text{ g ethanol / g glucose}$$

For CO_2, the maximum yield is

$$Y_{CO_2/S} = 2(44)/180 = 0.49 \text{ g } CO_2\text{/g glucose}$$

In practice, these maximum yields are not obtained. The product yields are about 90% to 95% of the maximum values because the glucose is converted into biomass and other metabolic by-products (e.g., glycerol or acetate).

7.4. ESTIMATION OF ELEMENTAL CELL COMPOSITION

Cells are made of C, N, O, H, P, S, and minerals (Ca, Mg, Fe, Zn, and others). Elemental composition of the cells varies depending on the type of cells (bacteria, yeasts, molds, animal and plant cells), type of substrate, and growth conditions (T, pH, ORP, DO). A typical bacterial cell contains nearly 45% to 50% C, 10% to 14% N, 20% to 25% O, 6% to 8% H, 2% to 3% P, 0.5% to 1% S, and 5% to 6% ash and is usually symbolized by $C_5H_7NO_2$ with a molar weight of 113 g/mol bacteria. However, this composition varies depending on the composition of growth medium (C, N, P, O sources) and environmental conditions.

Elemental composition of the cells needs to be determined before balancing biological reactions. This determination is done by filtering and drying the cells at 105°C until constant weight. Elemental analyses are carried out on dry cell mass to determine the percentages of C, N, O, H, P, and S content. Dried cells are further burned in a furnace at 550°C to remove organic fractions

(volatile suspended solids) and to determine the ash content. The mass fraction of every element in dry cell mass is then converted to mole fractions to determine the empirical formula for one mole of cell.

The mole number of the *i*th element is

$$N_i = m_i / M_i \tag{7.15}$$

where N_i is the mole number, m_i is the weight, and M_i is the molar weight of the *i*th elements. The total number of moles is

$$N_T = \Sigma N_i = N_c + N_N + N_o + N_H + N_P + N_\Sigma \tag{7.16}$$

The mole fraction for the *i*th element is

$$X_i = N_i / N_T \tag{7.17}$$

Once the mole fractions are determined, the empirical formula is established as follows:

$C_{Xc}\, H_{XH}\, N_{XN}\, O_{XO}\, P_{XP}\, S_{XS}$

Example 7.3.

One gram of dry cell mass is analyzed for major elements, and the following results are obtained: m_C = 0.48g, m_H = 0.07 g, m_O = 0.24 g, m_N = 0.12 g, and m_{ash} = 0.09 g. Ash includes P, S, and minerals. Determine the empirical formula of the cell.

Solution Mole numbers of each element are as follows:

N_C = 0.48/12 = 0.04, N_H = 0.07 mole, N_O = 0.24/16 = 0.015, N_N = 0.12/14 = 0.0086 mole

Total number of moles (mole number of cell) N_T = 0.1336 mole.

Mole fractions of the elements are as follows:

X_C = 0.04/0.1336 = 0.30, X_H = 0.524, X_O = 0.112, X_N = 0.064

Then the following is the empirical formula of the cell:

$C_{0.3}\, H_{0.524}\, O_{0.112}\, N_{0.064}$ or $C_{4.68}\, H_{8.2}\, O_{1.75}\, N$

7.5. STOICHIOMETRY BY OXIDATION-REDUCTION HALF-REACTIONS

In biological conversions, a substrate such as glucose is both the source of energy and the material used for biosynthesis of cells and the products. A fraction of the total electrons present in the substrate is transferred to the electron acceptor by metabolic reactions to generate energy in the form of ATPs (e_e), and the remaining fraction ends up in the newly synthesized cellular mass (e_s). The sum of e_e and e_s is 1.0. When electrons are transferred from the substrate to the electron acceptor, the net reaction can be represented by two half-reactions in the form of oxidation and reduction. The electron donor (substrate) is oxidized, and the electron acceptor is reduced. In aerobic oxidation of glucose to CO_2 and H_2O, glucose-carbon is the electron donor and oxygen is the electron acceptor. The electron donor (C in glucose) is oxidized to CO_2 by donating electrons to the electron acceptor (O_2), yielding water. This bioconversion can be represented by the following half-reactions based on one mole electron transfer:

$$\textit{Oxidation}: \quad 1/24\ C_6H_{12}O_6 + \tfrac{1}{4}\ H_2O \rightarrow \tfrac{1}{4}\ CO_2 + H^+ + e^- \qquad (7.18)$$

$$\textit{Reduction}: \quad 1/4\ O_2 + H^+ + e^- \rightarrow \tfrac{1}{2}\ H_2O \qquad (7.19)$$

$$\textit{Overall}: \quad 1/24\ C_6H_{12}O_6 + \tfrac{1}{4}\ O_2 \rightarrow \tfrac{1}{4}\ H_2O + \tfrac{1}{4}\ CO_2 \qquad (7.20)$$

By multiplying both sides of the overall reaction by 24, the overall reaction is obtained:

$$C_6H_{12}O_6 + 6\ O_2 \rightarrow 6\ CO_2 + 6\ H_2O \qquad (7.21)$$

A list of half-reactions for common electron donors is presented in Table 7.5. Electron acceptor is oxygen in aerobic respiration. However, nitrate, sulfate, or Fe (III) may be the electron acceptor in anaerobic respiration. Carbon dioxide is the electron acceptor in anaerobic metabolism of carbonaceous compounds yielding methane as the end product. Table 7.6 summarizes half-reactions for common electron acceptors.

Compound	Half-Reaction	ΔG° (kJ/eq e^-)
Glucose	$1/24\ C_6H_{12}O_6 + ¼\ H_2O \rightarrow ¼\ CO_2 + H^+ + e^-$	−41.35
Glycerol	$1/14\ C_3H_5(OH)_3 + 3/14\ H_2O \rightarrow 3/14\ CO_2 + H^+ + e^-$	−38.88
Ethanol	$1/12\ C_2H_5OH + ¼\ H_2O \rightarrow 1/6\ CO_2 + H^+ + e^-$	−31.18
Methanol	$1/6\ CH_3OH + 1/6\ H_2O \rightarrow 1/6\ CO_2 + H^+ + e^-$	−36.84
Methane	$1/8\ CH_4 + ¼\ H_2O \rightarrow 1/8\ CO_2 + H^+ + e^-$	−23.53
Acetate	$1/8\ CH_3COO^- + 3/8\ H_2O \rightarrow 1/8\ CO_2 + 1/8\ HCO_3^- + H^+ + e^-$	−27.40
Benzoate	$1/30\ C_6H_5COO^- + 13/30\ H_2O \rightarrow 1/5\ CO_2 + 1/30\ HCO_3^- + H^+ + e^-$	−27.34
Lactate	$1/12\ CH_3CHOHCOO^- + 1/3\ H_2O \rightarrow 1/6\ CO_2 + 1/12\ HCO_3^- + H^+ + e^-$	−32.29
Propionate	$1/14\ CH_3CH_2COO^- + 5/14\ H_2O \rightarrow 1/7\ CO_2 + 1/14\ HCO_3^- + H^+ + e^-$	−27.63
Pyruvate	$1/10\ CH_3COCOO^- + 2/5\ H_2O \rightarrow 1/5\ CO_2 + 1/10\ HCO_3^- + H^+ + e^-$	−35.09
Cell Synthesis	$1/20\ C_5H_7NO_2 + 9/20\ H_2O \rightarrow 1/5\ CO_2 + 1/20\ NH_4^+ + 1/20\ HCO_3^- + H^+ + e^-$	

Adapted from Rittmann & McCarty, *Environmental Biotechnology*, McGraw Hill, 2001.

Table 7.5. Major Electron-Donor Half-Reactions

Compound	Half-Reaction	ΔG° (kJ/eq e^-)
Oxygen	$¼\ O_2 + H^+ + e^- \rightarrow ½\ H_2O$	−78.72
Nitrate	$1/5\ NO_3^- + 6/5\ H^+ + e^- \rightarrow 1/10\ N_2 + 3/5\ H_2O$	−72.20
Sulfate	$1/8\ SO_4^{-2} + 19/16\ H^+ + e^- \rightarrow 1/16\ H_2S + 1/16\ HS^- + 1/2\ H_2O$	−20.85
Fe(III)	$Fe(III) + e^- \rightarrow Fe(II)$	−74.27
CO_2	$1/8\ CO_2 + H^+ + e^- \rightarrow 1/8\ CH_4 + ¼\ H_2O$	23.53

Adapted from Rittmann & McCarty, *Environmental Biotechnology*, McGraw Hill, 2001.

Table 7.6. Major Electron-Acceptor Half-Reactions

As mentioned before, a fraction of electrons present in the substrate ends up in the newly synthesized biomass. The empirical formula for bacterial cell is usually assumed to be $C_5H_7NO_2$ which varies depending on the type of cell (yeasts, molds, animal and plant cells) and medium used. As shown in Section 7.4 it is possible to determine a more exact formula for a particular culture. Considering inorganic carbon (CO_2 and HCO_3^-) as carbon and NH_4^+ as nitrogen source, the half-reaction for cell synthesis can be written as follows for one mole electron transfer:

$$\frac{1}{5}CO_2 + \frac{1}{20}NH_4^+ + \frac{1}{20}HCO_3^- + H^+ + e^- \rightarrow \frac{1}{20}C_5H_7NO_2 + \frac{9}{20}H_2O \qquad (7.22)$$

This is a reduction half-reaction where inorganic carbon is the electron acceptor. When nitrate is used as the N-source, the half-reaction for cell synthesis can be written as follows:

$$\frac{1}{28}NO_3^- + \frac{5}{28}CO_2 + \frac{29}{28}H^+ + e^- \rightarrow \frac{1}{28}C_5H_7NO_2 + \frac{11}{28}H_2O \quad (7.23)$$

Cell synthesis half-reactions are the electron-acceptor reactions that must accompany an electron-donor reaction such as the reaction shown in equation 7.18.

The fraction of electrons used for energy generation (e_e) and for cell synthesis (e_s) need to be known in balancing microbial reactions. Net energy generation and net biosynthesis reactions are

$$R_e = R_a + R_d \text{ and } R_s = R_c + R_d \quad (7.24)$$

where R_e and R_s are the net energy generation and cell synthesis reactions and R_a, R_d, and R_c are the electron acceptor, electron donor, and cell synthesis (electron acceptor) half-reactions, respectively.

The net reaction based on fractional electron utilization is

$$R = e_e R_e + e_s R_s = e_e (R_a + R_d) + e_s (R_c + R_d) \quad (7.25)$$

Since $e_e + e_s = 1$, equation 7.25 can be written as

$$R = e_e R_a + e_s R_c + R_d \quad (7.26)$$

Equation 7.25 or 7.26 can be used in balancing microbial reactions.

Example 7.4.

Aerobic oxidation of glucose is accompanied by microbial growth. NH_4^+ is used as the nitrogen source and the end products are CO_2 and H_2O. The formula for bacterial cell is $C_5H_7NO_2$. Determine the coefficients for this microbial conversion. Assume that 40% of total electrons are used for biosynthesis and 60% are used for energy generation.

Solution

Following are half-reactions for microbial oxidation of glucose:

Electron-donor reaction (R_d): $1/24\ C_6H_{12}O_6 + ¼\ H_2O \rightarrow ¼\ CO_2 + H^+ + e^-$

Electron-acceptor reaction (R_a): $1/4\ O_2 + H^+ + e^- \rightarrow ½\ H_2O$

The half-reaction for cell synthesis (R_c) is

$$\frac{1}{5}CO_2 + \frac{1}{20}NH_4^+ + \frac{1}{20}HCO_3^- + H^+ + e^- \rightarrow \frac{1}{20}C_5H_7NO_2 + \frac{9}{20}H_2O$$

The net energy generating reaction is

$$R_e = R_a + R_d = \frac{1}{24}C_6H_{12}O_6 + ¼\ O_2 \rightarrow ¼\ H_2O + ¼\ CO_2$$

The net cell synthesis reaction is

$$R_s = R_c + R_d = 1/24\ C_6H_{12}O_6 + \frac{1}{20}NH_4^+ + \frac{1}{20}HCO_3^- \rightarrow \frac{1}{20}C_5H_7NO_2 + \frac{1}{20}CO_2 + \frac{1}{5}H_2O$$

The overall reaction is

$$R = e_e R_e + e_s R_s = 0.60R_e + 0.40\ R_s = 0.0417C_6H_{12}O_6 + 0.02\ NH_4^+ + 0.15\ O_2 + 0.02HCO_3^- \rightarrow 0.02C_5H_7NO_2 + 0.17\ CO_2 + 0.23\ H_2O$$

or

$$C_6H_{12}O_6 + 0.48NH_4^+ + 3.6O_2 + 0.48HCO_3^- \rightarrow 0.48C_5H_7NO_2 + 4.08CO_2 + 5.52H_2O$$

The growth yield coefficient and the respiratory quotient for this reaction are $Y_{x/s}$ = 0.48 (113)/180 = 0.30 g cell/g glucose, and RQ = 4.08/3.6 = 1.33 mol CO_2/mol O_2.

7.6. THERMODYNAMICS OF BIOLOGICAL REACTIONS

Like chemical reactions, biological reactions obey thermodynamic laws too. Energy is conserved in all biological reactions. Spontaneous bioreactions result in negative free-energy changes. Therefore, by looking at the free-energy changes in bioreactions, it is possible to determine which reactions would take place spontaneously and which require energy input. Free-energy changes in bioreactions are calculated by using the formation free energies of products and reactants. In general, free-energy change in a reaction is calculated by using the following equation:

$$\Delta G = \Sigma\ a_i\ G_i - \Sigma\ b_j G_j \qquad (7.27)$$

where $\Sigma\ a_i\ G_i$ is the sum of free energies of the products, $\Sigma\ b_j G_j$ is the sum of free energies of reactants, and ΔG is the free-energy change of the reaction.

Free energy of any compound is defined as

$$G_i = G_i^o + RT\ LnC_i \qquad (7.28)$$

where G_i^o is the standard free energy (25°C, 1 atm), R is the gas constant (1.99 cal/mol-K), T is the absolute temperature (K), and C_i is the molar concentration of the ith compound in aqueous solution. Therefore, free-energy change for the following bioreaction is

$$aA + bB \rightarrow cC + dD \tag{7.29}$$

$$\Delta G = \Delta G^{o} + RT\ LnQ = \Delta G^{o} + RT\ Ln\frac{(C)^{c}(D)^{d}}{(A)^{a}(B)^{b}} \tag{7.30}$$

Usually, standard free energies are calculated from the half-reactions of the electron donors and acceptors to determine the energy requirement of a reaction.

For aerobic microbial oxidation of glucose, the electron-donor half-reaction is

$1/24\ C_6H_{12}O_6 + ¼\ H_2O \rightarrow ¼\ CO_2 + H^+ + e^-$

The standard free-energy change for this reaction is

$$\Delta G^{o}_{d} = ¼\ G^{o}_{CO2} + G^{o}_{H+} - 1/24\ G^{o}_{Glu} - ¼\ G^{o}_{H2O}$$

$$= ¼(-394.36) + (-39.87) - 1/24(-917.22) - 1/4(-237.18) = -40.956\ \text{kJ/eq e}^-$$

The electron-acceptor half-reaction for the same bioconversion is

$$1/4\ O_2 + H^+ + e^- \rightarrow ½\ H_2O$$

The standard free-energy change for this reaction is

$$\Delta G^{o}_{a} = 1/2G^{o}_{H2O} - G^{o}_{H+} - 1/4G^{o}_{O2} = ½(-237.18) - (-39.87) - ¼(0) = -78.72\ \text{kJ/eq e}^-$$

Therefore, the free-energy change for the net reaction is

$1/24\ C_6H_{12}O_6 + ¼\ H_2O \rightarrow ¼\ CO_2 + H^+ + e^-$ $\quad \Delta G^{o}_{d} = -40.96\ \text{kJ/ eq e}^-$

$1/4\ O_2 + H^+ + e^- \rightarrow ½\ H_2O$ $\quad \Delta G^{o}_{a} = -78.72\ \text{kJ/ eq e}^-$

$1/24\ C_6H_{12}O_6 + ¼\ O_2 \rightarrow ¼\ CO_2 + ¼\ H_2O$ $\quad \Delta G^{o} = -119.68\ \text{kJ/ eq e}^-$

Therefore, this bioreaction is spontaneous and energetically favorable. Free-energy changes for different electron donor and acceptors have been calculated. Figure 7.1 depicts variation of free-energy changes for different electron donors and acceptors. When glucose is used as the electron donor and oxygen as the electron acceptor in a bioreaction, the free-energy change is around –120 kJ/eq e^-, whereas with the nitrate as the electron acceptor the free-energy change becomes nearly –110 kJ/eq e^-. The free-energy change is much lower when sulfate is used as the electron acceptor (about –20kJ/eq e^-). Under anaerobic conditions, where glucose is the electron donor and CO_2 is the acceptor, the free-energy change is –18 kJ/eq e^-. The difference between the free-energy

changes of the same reaction under aerobic and anaerobic conditions is the reason for differences in growth yield coefficients. Large free-energy changes in aerobic metabolism of glucose result in high aerobic-growth-yield coefficients as compared to that of anaerobic growth.

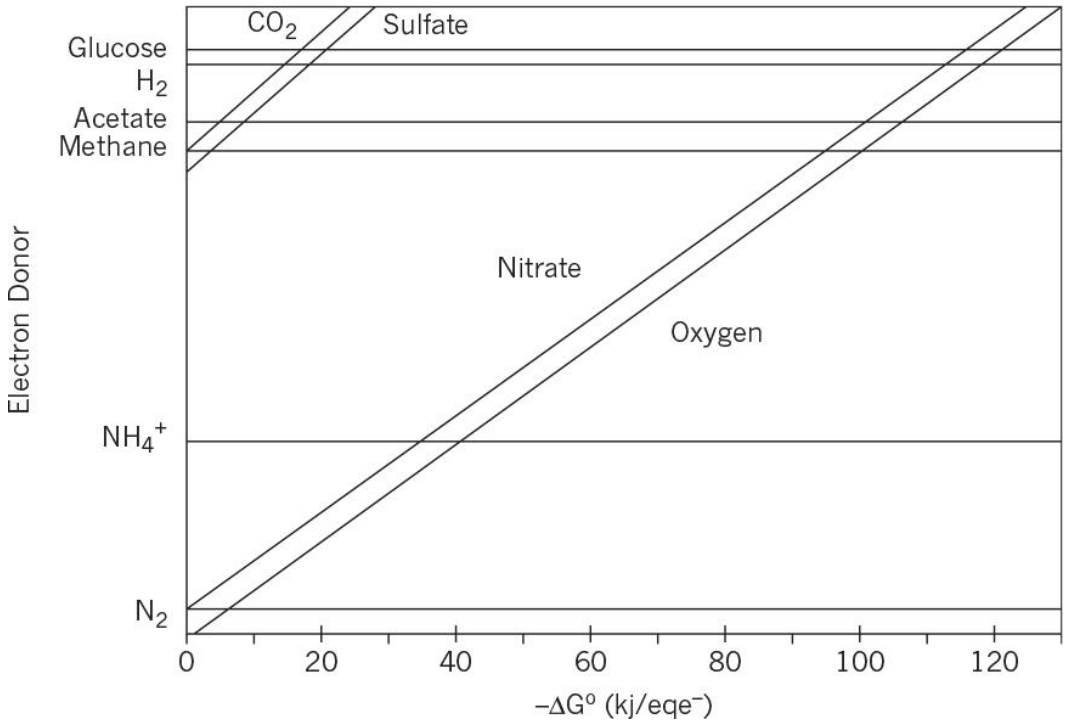

FIGURE 7.1. Variation of reaction free energies for different electron donors and acceptors. The lines represent electron acceptors. (Adapted from Rittmann & McCarty, *Environmental Biotechnology*, McGraw Hill, 2001.)

7.7. SUMMARY

This chapter reviewed simple methods to determine the reaction stoichiometry for bioreactions. These methods lead to the possibility of predicting yield coefficients for various fermentations using a variety of substrates. By coupling these equations to experimentally measurable parameters, such as the respiratory quotient, we can infer a great deal about the progress of fermentation. Such calculations can also assist in initial process design equations by allowing the prediction of the amount of oxygen required (and consequently heat generated) for a certain conversion of a particular substrate. The prediction of yield coefficients is usually not exact, because unknown or unaccounted metabolic pathways and products may be present. Nonetheless, such calculations provide useful first estimates of such parameters.

PROBLEMS

7.1. Determine the amount of $(NH_4)_2SO_4$ to be supplied in a fermentation medium where the final cell concentration is 30 g/l in a $10^3$1 culture volume. Assume that the cells are 12% nitrogen by weight and $(NH_4)_2SO_4$ is the only nitrogen source.

7.2. The growth of baker's yeast (*S. cerevisiae*) on glucose may be simply described by the following equation:

$$C_6H_{12}O_6 + 3\ O_2 + 0.48\ NH_3 \rightarrow \underset{\text{yeast}}{0.48\ C_6H_{10}NO_3} + 4.32\ H_2O + 3.12\ CO_2$$

In a batch reactor of volume 10^5 1, the final desired yeast concentration is 50 gdw/l.

a. Determine the concentration and total amount of glucose and $(NH_4)_2SO_4$ in the nutrient medium.

b. Determine the yield coefficients $Y_{X/S}$ (biomass/glucose) and $Y_{X/O}$ (biomass/oxygen).

c. Determine the total amount of oxygen required.

d. If the rate of growth at exponential phase is r_x = 0.7 gdw/l-h, determine the rate of oxygen consumption (g O_2/1-h)

e. Calculate the heat-removal requirements for the reactor (recall equation 6.26).

7.3. The growth of *S. cerevisiae* on glucose under anaerobic conditions can be described by the following overall reaction:

$$C_6H_{12}O_6 + \beta\ NH_3 \rightarrow 0.59\ CH_{1.74}N_{0.2}O_{0.45}\ (\text{biomass}) + 0.43\ C_3H_8O_3 + 1.54\ CO_2 + 1.3\ C_2H_5OH + 0.036\ H_2O$$

a. Determine the biomass yield coefficient $Y_{X/S}$.

b. Determine the product yield coefficients $Y_{EtOH/S}$, Y_{CO2}/S, $Y_{C3}H_2O/S$.

c. Determine the coefficient β.

7.4. Aerobic growth of *S. cerevisiae* on ethanol is simply described by the following overall reaction:

$$C_2H_5OH + a\ O_2 + b\ NH_3 \rightarrow c\ CH_{1.704}N_{0.149}O_{0.408} + d\ CO_2 + e\ H_2O$$

a. Determine the coefficients *a*, *b*, *c*, and *d*, where RQ = 0.66.

b. Determine the biomass yield coefficient, $Y_{X/S,}$ and oxygen yield coefficient, $Y_{X/O2}$ (gdw/g O_2).

7.5. Aerobic degradation of benzoic acid by a mixed culture of microorganisms can be represented by the following reaction:

$$\underset{\text{(substrate)}}{C_6H_5COOH} + a\,O_2 + b\,NH_3 \rightarrow \underset{\text{(bacteria)}}{c\,C_5H_7NO_2} + d\,H_2O + e\,CO_2$$

a. Determine *a*, *b*, *c*, *d*, and *e* if RQ = 0.9.

b. Determine the yield coefficients, $Y_{X/S}$ and $Y_{X/O2}$.

c. Determine degree of reduction for the substrate and bacteria.

7.6. Aerobic degradation of an organic compound by a mixed culture of organisms in waste water can be represented by the following reaction:

$C_3H_6O_3 + a\,O_2 + b\,NH_3 \rightarrow c\,C_5H_7NO_2 + d\,H_2O + e\,CO_2$

a. Determine *a*, *b*, *c*, *d*, and *e* if $Y_{X/S}$ = 0.4 g X/g S.

b. Determine the yield coefficients $Y_{X/O2}$ and $Y_{X/NH3}$

c. Determine the degree of reductions for the substrate, bacteria, and RQ for the organisms.

7.7. Biological denitrification of nitrate-containing waste waters can be described by the following overall reaction:

$$NO_3^{-1} + a\,CH_3OH + H^+ \rightarrow b\,C_5H_7NO_2 + c\,N_2 + d\,CO_2 + e\,H_2O$$

a. Determine *a*, *b*, *c*, *d*, and *e* if $Y_{X/S}$ = 0.5 g *X*/g N.

b. Determine the degree of reduction of bacteria and methanol.

7.8. The following biological oxidation reaction takes place in biological treatment of an industrial wastewater:

$C_6H_4CH_3OH + a\,O_2 + b\,NH_3 \rightarrow c\,C_5H_7NO_2 + d\,CO_2\ e\,H_2O$

a. Determine the coefficients of the equation (*a*, *b*, *c*, *d*, and *e*) if RQ = 0.5.

b. Determine $Y_{x/s}$, and $Y_{X/O2}$.

c. Determine γ_s and γ_b.

7.9. *Clostridium* sp. were used for anaerobic conversion of glucose to acetic acid and H_2 as described by the following reaction:

$C_6H_{12}O_6 + a\ NH_3 + b\ H_3PO_4 \rightarrow c\ C_5H_7NO_2P_{0.2} + d\ CH_3COOH + e\ CO_2 + f\ H_2$

a. Determine the coefficients by writing elemental balances if $Y_{x/s}$ = 0.1 g/g.

b. Determine γ_s, γ_b, γ_{Ac}, and γ_{H2} and write down degree of reduction balance to see if it is satisfied.

c. Determine $Y_{p/s}$ for acetic acid and hydrogen.

7.10. *Rhodobacter* sp. ferments acetic acid to hydrogen gas and CO_2 under photoheterotrophic conditions (anoxygenic photosynthesis) as depicted in the following reaction:

$$CH_3COOH + a\ NH_3 + b\ H_3PO_4 \xrightarrow{\text{Light}} c\ C_5H_7NO_2P_{0.2} + d\ CO_2 + e\ H_2$$

a. Determine the coefficients by using elemental balances.

b. Determine the growth yield coefficient $Y_{x/s}$ and $Y_{p/s}$.

c. Write down degree of reduction balance for the conversion.

7.11. Elemental analysis of dry cell mass indicates that the cell is composed of 45% C, 25% O, 5% H, 12% N, 2.5% P, 0.5% S, and 10% minerals.

a. By considering 1 g of cell dry weight, determine mole fractions of every element and the cell formula.

b. If the cell formula is $C_4\ H_8\ O_{2.2}\ N_{1.2}\ P_{0.08}\ S_{0.02}$, determine the percent elemental composition (i.e., mass fractions) of dry cell mass.

7.12. In ethanol fermentation from glucose, carbon dioxide is the electron acceptor, and glucose is the electron donor.

a. Write down the electron donor and acceptor half-reactions for ethanol fermentation for one mole electron transfer along with free-energy changes.

b. Determine the overall fermentation reaction for one mole of glucose and the free-energy change without cell-synthesis reaction.

c. Assuming 20% of the electrons in glucose are used for cell synthesis and 80% for energy generation (e_e = 0.80 and e_s = 0.20), write down the reactions for Ra, Rd, and Rc, and determine the overall reaction stoichiometry for one mole of NH_4-N.

7.13. In biological nitrification reaction NH_4-N is converted to NO_3-N under autotrophic conditions by using CO_2 as the carbon source in the presence of oxygen. NH_4-N is the electron

donor, and oxygen is the electron acceptor in this bioconversion, which is realized in the absence of organic carbon source. Ammonium is also used as N-source for cell synthesis. If 85% of electrons are transferred to the product for energy generation and only 15% ends up in cell biomass, determine the following:

a. Write down the electron donor and acceptor half-reactions (R_d and R_a).

b. Write down the half-reaction for cell synthesis (R_c).

c. Write down the overall reaction by considering the electron fractionation.

7.14. Methane formation from acetate is realized under anaerobic conditions where NH_4 is the nitrogen source for cell synthesis. Acetate is the electron donor and CO_2 is the electron acceptor in this bioconversion. Assuming the cell formula is $C_5H_7NO_2$ and only 10% of the electrons in the substrate are transferred to the cell biomass, determine the following:

a. Write down the electron donor and the acceptor half-reactions (R_d and R_a).

b. Write down the cell synthesis half-reaction (R_c).

c. Write down the overall reaction by considering that $e_e = 0.90$ and $e_s = 0.10$.

7.15. Determine the free-energy changes for methane formation from the electron donors of glucose, acetate, H_2 and ethanol where CO_2 is the electron acceptor by using Figure 7.1. Comment on the results in terms of cell yield from different substrates.

Degree of Reduction

In more complex reactions, as in the formation of extracellular products, an additional stoichiometric coefficient is added, requiring more information. Also, elemental balances provide no insight into the energetics of a reaction. Consequently, the concept of degree of reduction has been developed and used for proton–electron balances in bioreactions. The degree of reduction, g, for organic compounds may be defined as the number of equivalents of available electrons per gram atom C. The available electrons are those that would be transferred to oxygen upon oxidation of a compound to CO2, H2O, and NH3. The degrees of reduction for some key elements are C = 4, H = 1, N = -3, O = -2, P = 5, and S = 6. The degree of reduction of any element in a compound is equal to the valence of this element. For example, 4 is the valence of carbon in CO2 and -3 is the valence of N in NH3. Degrees of reduction for various organic compounds are given in Table 7.4. The following are examples of how to calculate the degree of reduction for substrates.

Methane (CH4): 1(4) + 4(1) = 8, g = 8/1 = 8

Glucose (C6H12O6): 6(4) + 12(1) + 6(-2) = 24, g = 24/6 = 4

Ethanol (C2H5OH): 2(4) + 6(1) + 1(-2) = 12, g = 12/2 = 6

A high degree of reduction indicates a low degree of oxidation. That is,
$\gamma_c CH4 > \gamma_c EtOH > \gamma_c glucose$.

Consider the aerobic production of a single extracellular product.

$$\underset{\text{substrate}}{CH_mO_n} + aO_2 + bNH_3 \longrightarrow \underset{\text{biomass}}{cCH_\alpha O_\beta N_\delta} + \underset{\text{product}}{dCH_xO_yN_z} + eH_2O + fCO_2 \qquad (7.6)$$

The degrees of reduction of substrate, biomass, and product are

$$\gamma_s = 4 + m - 2n \qquad (7.7)$$

TABLE 7.4 Degree of Reduction and Weight of One Carbon Equivalent of One Mole of Some Substrates and Biomass

Compound	Molecular Formula	Degree of Reduction, γ	Weight, *m*
Biomass	$CH_{1.64}N_{0.16}O_{0.52}P_{0.0054}S_{0.005}$[a]	4.17 (NH_3) 4.65 (N_2) 5.45 (HNO_3)	24.5
Methane	CH_4	8	16.0
n-Alkane	$C_{15}H_{32}$	6.13	14.1
Methanol	CH_4O	6.0	32.0
Ethanol	C_2H_6O	6.0	23.0
Glycerol	$C_3H_8O_3$	4.67	30.7
Mannitol	$C_6H_{14}O_6$	4.33	30.3
Acetic acid	$C_2H_4O_2$	4.0	30.0
Lactic acid	$C_3H_6O_3$	4.0	30.0
Glucose	$C_6H_{12}O_6$	4.0	30.0
Formaldehyde	CH_2O	4.0	30.0
Gluconic acid	$C_6H_{12}O_7$	3.67	32.7
Succinic acid	$C_4H_6O_4$	3.50	29.5
Citric acid	$C_6H_8O_7$	3.0	33.5
Formic acid	CH_2O_2	2.0	46.0
Oxalic acid	$C_2H_2O_4$	1.0	45.0

$$\gamma_b = 4 + \alpha - 2\beta - 3\delta \tag{7.8}$$

$$\gamma_p = 4 + x - 2y - 3z \tag{7.9}$$

Note that for CO2, H2O, and NH3 the degree of reduction is zero.

Equation 7.6 can lead to elemental balances on C, H, O, and N, an available electron balance, an energy balance, and a total mass balance. Of the equations, only five will be independent. If all the equations are written, then the extra equations can be used to check the consistency of an experimental data set. Because the amount of water formed or used in such reactions is difficult to determine and water is present in great excess, the hydrogen and oxygen balances are difficult to use. For such a data set, we would typically choose a carbon, a nitrogen, and an available-electron balance. Thus,

$$c + d + f = 1 \tag{7.10}$$

$$c\delta + dz = b \tag{7.11}$$

$$c\gamma_b + d\gamma_p = \gamma_s - 4a \tag{7.12}$$

With partial experimental data, it is possible to solve this set of equations. Measurements of RQ and a yield coefficient would, for example, allow the calculation of the remaining coefficients. It should be noted that the coefficient, *c*, is *YX*/*S* (on a molar basis) and *d* is *YP*/*S* (also on a molar basis). An energy balance for aerobic growth is

$$Q_0 c\gamma_b + Q_0 d\gamma_p = Q_0\gamma_s - Q_0 4a \quad (7.13)$$

If *Q*0, the heat evolved per equivalent of available electrons transferred to oxygen, is constant, eq. 7.13 is *not* independent of eq. 7.12. Recall that an observed regularity is 26.95 kcal/g equivalent of available electrons transferred to oxygen, which allows the prediction of heat evolution based on estimates of oxygen consumption. Equations 7.12 and 7.13 also allow estimates of the fractional allocation of available electrons or energy for an organic substrate. Equation 7.12 can be rewritten as

$$1 = \frac{c\gamma_b}{\gamma_s} + \frac{d\gamma_p}{\gamma_s} + \frac{4a}{\gamma_s} \quad (7.14a)$$

$$1 = \xi_b + \xi_p + \varepsilon \quad (7.14b)$$

where e is the fraction of available electrons in the organic substrate that is transferred to oxygen, x*b* is the fraction of available electrons that is incorporated into biomass, and x*p* is the fraction of available electrons that is incorporated into extracellular products.

Example 7.1

Assume that experimental measurements for a certain organism have shown that cells can convert two-thirds (wt/wt) of the substrate carbon (alkane or glucose) to biomass.

a. Calculate the stoichiometric coefficients for the following biological reactions:

Hexadecane: C16H34 + *a* O2 + *b* NH3 aA *c*(C4.4H7.3N0.86O1.2) + *d* H2O + *e* CO2

Glucose: C6H12O6 + *a* O2 + *b* NH3 aA *c*(C4.4H7.3N0.86O1.2) + *d* H2O + *e* CO2

b. Calculate the yield coefficients *YX/S* (g dw cell/g substrate), *YX/O*2 (g dw cell/g O2) for both reactions. Comment on the differences.

Solution

a. For hexadecane,

amount of carbon in 1 mole of substrate = 16(12) = 192 g

amount of carbon converted to biomass = 192(2/3) = 128 g

Then, 128 = c(4.4)(12); c = 2.42.

amount of carbon converted to CO2 = 192 - 128 = 64 g

64 = e (12), e = 5.33

The nitrogen balance yields

14b = c(0.86)(14)

b = (2.42)(0.86) = 2.085

The hydrogen balance is

34(1) + 3b = 7.3c + 2d

d = 12.43

The oxygen balance yields

2a(16) = 1.2c(16) + 2e(16) + d(16)

a = 12.427

For glucose,

amount of carbon in 1 mole of substrate = 72 g

amount of carbon converted to biomass = 72(2/3) = 48 g

Then, 48 = 4.4c(12); c = 0.909.

amount of carbon converted to CO2 = 72 - 48 = 24 g

24 = 12e; e = 2

The nitrogen balance yields

14b = 0.86c(14)

b = 0.782

The hydrogen balance is

12 + 3b = 7.3c + 2d

d = 3.854

The oxygen balance yields

6(16) + 2(16)a = 1.2(16)c + 2(16)e + 16d

a = 1.473

b. For hexadecane,

$$Y_{X/S} = \frac{2.42(\mathrm{MW})_{\text{biomass}}}{(\mathrm{MW})_{\text{substrate}}}$$

$$Y_{X/S} = \frac{2.42(91.34)}{226} = 0.98 \text{ gdw cells/g substrate}$$

$$Y_{X/O_2} = \frac{2.42(\mathrm{MW})_{\text{biomass}}}{12.43(\mathrm{MW})_{O_2}}$$

$$Y_{X/O_2} = \frac{2.42(91.34)}{(12.43)(32)} = 0.557 \text{ gdw cells/g } O_2$$

For glucose,

$$Y_{X/S} = \frac{(0.909)(91.34)}{180} = 0.461 \text{ gdw cells/g substrate}$$

$$Y_{X/O_2} = \frac{(0.909)(91.34)}{(1.473)(32)} = 1.76 \text{ gdw cells/g } O_2$$

The growth yield on more reduced substrate (hexadecane) is higher than that on partially oxidized substrate (glucose), assuming that two-thirds of all the entering carbon is incorporated in cellular structures. However, the oxygen yield on glucose is higher than that on the hexadecane, since glucose is partially oxidized.

THEORETICAL PREDICTIONS OF YIELD COEFFICIENTS

In aerobic fermentations, the growth yield per available electron in oxygen molecules is approximately 3.14 ±0.11 gdw cells/electron when ammonia is used as the nitrogen source. The number of available electrons per oxygen molecule (O2) is four. When the number of oxygen molecules per mole of substrate consumed is known, the growth yield coefficient, *YX/S*, can easily be calculated. Consider the aerobic catabolism of glucose.

C6H12O6 + 6 O2 aA 6 CO2 + 6 H2O

The total number of available electrons in 1 mole of glucose is 24. The cellular yield per available electron is *YX/S* = 24(3.14) = 76 gdw cells/mol.

The predicted growth yield coefficient is *YX/S* = 76/180 = 0.4 gdw cells/g glucose.

Most measured values of *YX/S* for aerobic growth on glucose are 0.38 to 0.51 g/g (see

Table 6.1).

The ATP yield (*YX*/ATP) in many anaerobic fermentations is approximately 10.5 $\pm$ 2 gdw cells/mol ATP. In aerobic fermentations, this yield varies between 6 and 29. When the energy yield of a metabolic pathway is known (*N* moles of ATP produced per gram of substrate consumed), the growth yield *YX/S* can be calculated using the following equation:

YX/S = *YX*/ATP *N*

Example 7.2

Estimate the theoretical growth and product yield coefficients for ethanol fermentation by *S. cerevisiae* as described by the following overall reaction:

$C_6H_{12}O_6 \longrightarrow 2\ C_2H_5OH + 2\ CO_2$

Solution Since *YX*/ATP a 10.5 gdw/mol ATP and since glycolysis yields 2 ATP/mol of glucose in yeast,

$$Y_{X/S} = 10.5\ \text{gdw/mol ATP} \cdot 2\frac{\text{moles ATP}}{180\ \text{g glucose}}$$

or

YX/S a 0.117 gdw/g glucose

For complete conversion of glucose to ethanol by the yeast pathway, the maximal yield would be

$$Y_{P/S} = \frac{2(46)}{180} = 0.51\ \text{g ethanol/g glucose}$$

while for CO2 the maximum yield is

$$Y_{CO_2/S} = \frac{2(44)}{180} = 0.49\ \text{g ethanol/g glucose}$$

In practice, these maximal yields are not obtained. The product yields are about 90% to 95% of the maximal values, because the glucose is converted into biomass and other metabolic by-products (e.g., glycerol or acetate).

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MICROBIAL GROWTH KINETICS

MICROBIAL GROWTH AND ITS QUANTIFICATION

1 Introduction

Growth is an orderly increase in the quantity of cellular constituents. It depends upon the ability of the cell to form new protoplasm from nutrients available in the environment. In most bacteria, growth involves increase in cell mass and number of ribosomes, duplication of the bacterial chromosome, synthesis of new cell wall and plasma membrane, partitioning of the two chromosomes, septum formation, and cell division. This asexual process of reproduction is called **binary fission**. For unicellular organisms such as bacteria, growth can be measured in terms of two different parameters: changes in **cell mass** and changes in **cell numbers**.

.2 Methods for Measurement of Cell Biomass

Methods for the measurement of the cell mass involve both direct and indirect techniques.

i. Direct **physical measurement** of dry weight, wet weight, or volume of cells after centrifugation.

ii. Direct **chemical measurement** of some chemical component of the cells such as total N, total protein, or total DNA contents.

iii. Indirect **measurement of chemical activity** such as rate of O_2 production or consumption, CO_2 production or consumption, etc.

iv. **Turbidity measurements** employ a variety of instruments to determine the amount of light scattered by a suspension of cells. Particulate objects such as bacteria scatter light in proportion to their numbers. The turbidity or **optical density** of a suspension of cells is directly related to cell mass or cell number. The method is simple and nondestructive, but the sensitivity is limited to about 10^7 cells per ml for most bacteria.

3.3 Methods for Measurement of Cell Numbers

Measuring techniques involve direct counts, visually or instrumentally, and indirect viable cell counts.

3.3.1 Direct microscopic counts (DMC)

DMC are possible using special slides known as counting chambers. Dead cells cannot be distinguished from living ones. Only dense suspensions can be counted ($>10^7$ cells per ml), but samples can be concentrated by centrifugation or filtration to increase sensitivity.

A variation of the direct microscopic count has been used to observe and measure growth of bacteria in natural environments. In order to detect and prove that thermophilic bacteria were growing in boiling hot springs, T.D. Brock immersed microscope slides in the springs and withdrew them periodically for microscopic observation. The bacteria in the boiling water attached to the glass slides naturally and grew as micro-colonies on the surface.

3.3.2 Electronic counting chambers

This is done to measure size distribution of cells. F or cells size of the bacteria, the suspending medium must be very clean. Such electronic devices are more often used to count eucaryotic cells such as blood cells.

3.3.3 Indirect viable cell counts

This is also called **plate counts**, involve plating out (spreading) a sample of a culture on a nutrient agar surface. The sample or cell suspension can be diluted in a nontoxic diluent (e.g. water or saline) before plating. If plated on a suitable medium, each viable unit grows and forms a colony. Each colony that can be counted is called a **colony forming unit (cfu)** and the number of cfu's is related to the viable number of bacteria in the sample.

Advantages of the technique are its sensitivity (theoretically, a single cell can be detected), and it allows for inspection and positive identification of the organism counted. Disadvantages are (1) only living cells develop colonies that are counted; (2) clumps or chains of cells develop into a single colony; (3) colonies develop only from those organisms for which the cultural conditions are suitable for growth. The latter makes the technique virtually useless to characterize or count the **total number of bacteria** in complex microbial ecosystems such as soil or the animal rumen or gastrointestinal tract. Genetic probes can be used to demonstrate the diversity and relative abundance of procaryotes in such an environment, but many species identified by genetic techniques have so far proven unculturable.

3.4 The Bacterial Growth Curve

In the laboratory, under favorable conditions, a growing bacterial population doubles at regular intervals. Growth is by geometric progression: 1, 2, 4, 8, etc. or 2^0, 2^1, 2^2, 2^3.....2^n **exponential growth**. In reality, exponential growth is only part of the bacterial life cycle, and not representative of the normal pattern of growth of bacteria in nature. (where n = the number of generations). This is called

When a fresh medium is inoculated with a given number of cells, and the population growth is monitored over a period of time, plotting the data will yield a **typical bacterial growth curve**

When bacteria are grown in a closed system (also called a batch culture), like a test tube, the population of cells almost always exhibits these growth dynamics: cells initially adjust to the new medium (lag phase) until they can start dividing regularly by the process of binary fission (exponential phase). When their growth becomes limited, the cells stop dividing (stationary phase), until eventually they show loss of viability (death phase). Note the parameters of the x and y axes. Growth is expressed as change in the number viable cells vs time. Generation times are calculated during the exponential phase of growth. Time measurements are in hours for bacteria with short generation times.

3.4.1 Four phases of the growth cycle

3.4.1.1 Lag phase

Immediately after inoculation of the cells into fresh medium, the population remains temporarily unchanged. Although there is no apparent cell division occurring, the cells may be growing in volume or mass, synthesizing enzymes, proteins, RNA, etc., and increasing in metabolic activity. The length of the lag phase is apparently dependent on a wide variety of factors including the size of the inoculum; time necessary to recover from physical damage or shock in the transfer; time required for synthesis of essential coenzymes or division factors; and time required for synthesis of new (inducible) enzymes that are necessary to metabolize the substrates present in the medium.

3.4.1.2 Exponential (log) phase

The exponential phase of growth is a pattern of balanced growth wherein all the cells are dividing regularly by binary fission, and are growing by geometric progression. The cells divide at a constant rate depending upon the composition of the growth medium and the conditions of incubation. The rate of exponential growth of a bacterial culture is expressed as **generation time**, also the **doubling time** of the bacterial population. Generation time (G) is defined as the time (t) per generation (n = number of generations). Hence, G=t/n is the equation from which calculations of generation time derive.

3.4.1.3 Stationary phase

Exponential growth cannot be continued forever in a **batch culture** (e.g. a closed system such as a test tube or flask). Population growth is limited by one of the three factors *viz.*, 1. exhaustion of

available nutrients; 2. accumulation of inhibitory metabolites or end products; 3. exhaustion of space, in this case called a lack of "biological space".

During the stationary phase, if viable cells are being counted, it cannot be determined whether some cells are dying and an equal number of cells are dividing, or the population of cells has simply stopped growing and dividing. The stationary phase, like the lag phase, is not necessarily a period of quiescence. Bacteria that produce **secondary metabolites**, such as antibiotics, do so during the stationary phase of the growth cycle (Secondary metabolites are defined as metabolites produced after the active stage of growth). It is during the stationary phase that spore-forming bacteria have to induce or unmask the activity of dozens of genes that may be involved in sporulation process.

3.4.1.4 Death phase

If incubation continues after the population reaches stationary phase, a death phase follows, in which the viable cell population declines. However, if counting is done by turbidimetric measurements or microscopic counts, the death phase cannot be observed. During the death phase, the number of viable cells decreases geometrically (exponentially), essentially the reverse of growth during the log phase.

3.5 Growth Rate and Generation Time

As mentioned above, bacterial growth rates during the phase of exponential growth, under standard nutritional conditions (culture medium, temperature, pH, etc.), define the bacterium's generation time. Generation times for bacteria vary from about 12 minutes to 24 hours or more. The generation time for *E. coli* in the laboratory is 15-20 minutes, but in the intestinal tract, the coliform's generation time is estimated to be 12-24 hours. For most known bacteria that can be cultured, generation times range from about 15 minutes to 1 hour. Symbionts such as *Rhizobium* tend to have longer generation times. Many lithotrophs, such as the nitrifying bacteria, also have long generation times. Some bacteria that are pathogens, such as *Mycobacterium tuberculosis* and *Treponema pallidum*, have especially long generation times, and this is thought to be an advantage in their virulence. Generation times for a few bacteria are shown in Table 3.1.

Table 3.1 Generation times for some common bacteria under optimal conditions of growth

3.5.1 Calculation of generation time

When growing exponentially by binary fission, the increase in a bacterial population is by geometric progression. If we start with one cell, when it divides, there are 2 cells in the first generation, 4 cells in the second generation, and 8 cells in the third generation, and so on. The **generation time** is the time interval required for the cells (or population) to divide.

G (generation time) = (time, in minutes or hours)/n(number of generations)

$G = t/n$

t = time interval in hours or minutes

B = number of bacteria at the beginning of a time interval

b = number of bacteria at the end of the time interval

n = number of generations (number of times the cell population doubles during the time interval)

$b = B \times 2^n$ (This equation is an expression of growth by binary fission)

Solve for n:

$\log b = \log B + n \log 2$

$$n = \frac{\log b - \log B}{\log 2}$$

$$n = \frac{\log b - \log B}{.301}$$

$n = 3.3 \log b / B$

$G = t/n$

Solve for G

$$G = \frac{t}{3.3 \log b/B}$$

3.6 Continuous Culture of Bacteria

The cultures so far discussed for growth of bacterial populations are called **batch cultures**. Since the nutrients are not renewed, exponential growth is limited to a few generations. Bacterial cultures can be maintained in a state of exponential growth over long periods of time using a system of **continuous culture** (Figure 2.2), designed to relieve the conditions that stop exponential growth in batch cultures. Continuous culture, in a device called a **chemostat or turbidostat** that

can be used to maintain a bacterial population at a constant density, a situation that is, in many ways, more similar to bacterial growth in natural environments.

Chemostat is a device for the continuous culture of bacteria. The chemostat relieves the environmental conditions that restrict growth by continuously supplying nutrients to cells and removing waste substances and spent cells from the culture medium.

In a chemostat, the growth chamber is connected to a reservoir of sterile medium. Once growth is initiated, fresh medium is continuously supplied from the reservoir. The volume of fluid in the growth chamber is maintained at a constant level by some sort of overflow drain. Fresh medium is allowed to enter into the growth chamber at a rate that limits the growth of the bacteria. The bacteria grow (cells are formed) at the same rate that bacterial cells (and spent medium) are removed by the overflow. The rate of addition of the fresh medium determines the rate of growth because the fresh medium always contains a limiting amount of an essential nutrient. Thus, the chemostat relieves the insufficiency of nutrients, the accumulation of toxic substances, and the accumulation of excess cells in the culture, which are the parameters that initiate the stationary phase of the growth cycle. The bacterial culture can be grown and maintained at relatively constant conditions, depending on the flow rate of the nutrients.

3.6.1 Synchronous growth of bacteria

Studying the growth of bacterial populations in batch or continuous cultures does not permit any conclusions about the growth behavior of individual cells, because the distribution of cell size (and hence cell age) among the members of the population is completely random. Information about the growth behavior of individual bacteria, however, is obtained by the study of **synchronous cultures**. Synchronized cultures must be composed of cells which are all at the same stage of the **bacterial cell cycle**. Measurements made on synchronized cultures are equivalent to measurements made on individual cells.

HEAT EVOLUTION DURING THE MICROBIAL PROCESS –

Introduction

The growth of microorganisms is accompanied by the production of heat, regardless of whether the system is aerobic or anaerobic or whether the final product is biomass or metabolites. A large part of the heat is generated during the degradation of the organic substrate, which serves as a carbon and energy source. The catabolic process is always associated with a significant free-energy decrease. Part of the released energy is conserved in high-energy bonds of adenosine triphosphate (ATP) or other energy-storage compounds, providing energy for biosynthesis of cellular components and other microbial activities as required. The rest of the substrate's original energy is released as heat. In addition to the heat production during the catabolism of the energy-rich nutrient molecules, most of the ATP energy is also liberated as heat during its utilization in the cellular activities, providing support for the microbial growth and other cell functions. The amount of heat produced is dependent upon the type of catabolic pathway through which the organic substrate is metabolized. It is also dependent upon the energetic coupling of energy-storage compound (ATP, etc.) generation and cell biosynthesis. Variations in the microbial heat parameter retie& the type of metabolic activities of the cell, as well as the degree of perfection with which the cellular metabolism and anabolism are completed. This indicates that the heat generation during the activity of microbial culture may be used to evaluate the regulatory mechanism of the cellular energy metabolism. It may also be very helpful in estimating the energetic efficiency of catabolic pathways, the heat energetic yield and the efficiency of energy recovery by the cells. However, the metabolic heat is still an under-utilized parameter for assessing the degree of microbial activities. This could be explained by the fact that the heat evolution is not usually monitored due to the difficulties and/or complexities involved in the measurement of such an elusive parameter. Also, there is a lack of good interpretation of the heat evolution quantity. In most cases, the rate of metabolic heat release has been correlated with the rate of oxygen consumption by the growing culture 1,2,3,~). This type of correlation is not applicable to anaerobic microbial systems and it only represents a narrow aspect of cellular metabolic activity

A successful attempt was reported by Belaich et al. 5) to correlate the quantity of heat released with the quantities of substrate utilization and product (ethanol) biosynthesis. The metabolic heat parameter has also been used to predict the maximum value of the specific rate of substrate

utilization and the Michaelis-Menten constant of the ethanol biosynthesis system. Many investigators have attempted the correlation between the heat evolution and cellular metabolic activity as a means of gaining insight into the thermodynamics and energetics of growth 6'7). However, little attention has been given to the possibility of measuring heat production for routine assessment of microbial kinetics. Mou and Cooney s), applying the dynamic calorimetric technique to an antibiotic biosynthesis in complex media, found thermal measurements useful for monitoring cell growth and as a physiological parameter throughout the bioconversion process. Recently, Wang et al. 9), Luong and Volesky 10), Volesky et al. 11) have used the parameter of heat produced to indirectly assess the concentration and rate of growth of cells during a microbial process. Heat and mass balances have been presented together with three regularities which Minkevich and Eroshin 12, 13, 14, 15) have identified and quantified. Erickson 16,17), and Erickson and co-workers ls,19,20,21) have applied the heat and mass balance regularities to specific culture conditions such as continuous cultivations, batch biosystems, and processes with extracellular products. The above-mentioned investigators have demonstrated that material and energy balance regularities can be used to analyze the process energetics. Applications of material and energy balances and associated regularities in on-line data analysis have been attempted 22). This approach is particularly important in on-line data analysis where only limited microbial process data are available. In view of this, the main purpose of this paper is to illustrate some important applications of the heat evolution quantity as a microbial process parameter. In aerobic systems, it can be used to predict the cellular metabolic activity, the biomass concentration, the oxygen uptake rate, and the organic substrate utilization rate. The heat evolution parameter can also be used, together with either oxygen uptake data or carbon dioxide respiration data, for checking the consistency of experimental data collected during the experiment. In anaerobic systems, the applicability of the heat evolution data as an analytical tool for the routine assessment of microbial kinetics will be discussed.

Factors Affecting Microbial Growth

The key to a productive staff is creating and maintaining a safe and healthy work environment. Maintaining work equipment and the physical state of a building is important, but there are potential threats lurking in a company's infrastructure that are too small for the naked eye to see. Fungi and other microbes often grow unchecked in the nooks and crannies of a building when potential problem areas with factors affecting microbial growth aren't identified and monitored regularly.

Fungi and bacteria cultivating in a building can enter the ventilation systems and cause widespread illness and discomfort among workers. With conditions like Legionellosis, or Legionnaires' Disease, and chronic upper respiratory problems as potential and persistent issues that could be caused by microbial growth, it's essential to know what kind of environmental conditions affect its growth and monitor areas of concern with the help of environmental consultants to keep air ventilation and water sources clean and free of health hazards.

THINGS TO CHECK TO IDENTIFY MICROBIAL GROWTH

Water and Dampness

Warmth, moisture, pH levels and oxygen levels are the four big physical and chemical factors affecting microbial growth. In most buildings, warmth and moisture are the biggest overall issues present. Dampness is a big player in the growth of fungi. Just like any living thing, water is essential to the life of microbes. They cannot multiply and spread without a consistent water source available to them. Bathrooms and basements tend to be prone to dampness and stagnant water, making them a key location for potential microbe issues. Leaks in the ceiling from rainfall or from pipes in the water system that aren't tended to can not only damage equipment but can also be breeding grounds for microbes in areas that are harder to notice or clean up.

Temperature

The temperature of an area can be a massive contributor to microbial growth. Bacteria thrives in warmth, growing the most in areas close to the temperature of a human's body. Cooler locations tend to slow growth of microbes, as seen when food is refrigerated to keep it safe to eat longer. Boiler rooms, rooms with heat-generating equipment and areas near heating vents can nest

bacteria and molds. Areas around machinery that create humidity are, of course, locations that can cause the most concern when one is minding the health of a building. Having an environmental consultant assess areas around equipment with heat as a byproduct can help you prevent potential health issues before they even develop by creating solutions through methods like improving airflow with optimized air ventilation.

Environmental pH

The pH level of an environment can either help or hurt the growth of microbes. Microbes tend to prefer pH levels that are neutral and are often harmed when more base or acidic elements are present in a location. It's for this reason that cleaning solutions, often highly acidic, kill off bacteria effectively when used. This means that improving cleaning regimens in a business's building can greatly reduce the number of microbes growing and deter future growths when performed regularly.

Oxygen and Nutrients

Oxygen-enriched locations and areas with vital nutrients will cultivate more microbial growth than locations with reduced oxygen levels. Controlling oxygen levels in an area can be difficult, but keeping areas clear of food and other sources of nutrients will starve out bacteria and keep a building clear of other pests as well.

Kinetic Studies on Cell Growth

1. Introduction

Cell growth implies increase in its mass and physical size controlled by physical, biological and chemical environments. Microbial growth is quantified by increase in the macromolecular and chemical constituents of the cell and growth pattern of each microbe is unique. Cell growth and cell division are inseparable for microbes as bacteria divide by binary fission, yeast cells by budding and viruses divide intracellularly. Microbial growth during log phase is very important for the analysis of cells due to division by binary fission. A typical mammalian cell growth is influenced by nutrient availability and thus a threshold cell size is required for DNA synthesis and mitosis. Thus, each class of organisms have a different growth pattern based on their cell cycle

and cell division. Understanding the growth kinetics of different classes of organisms forms the basis for fermentation process to achieve optimum product concentration.

Growth kinetics is an autocatalytic reaction which implies that the rate of growth is directly proportional to the concentration of cell. The cell concentration is measured by direct and indirect methods. Direct methods include measuring the cell mass concentration and cell number density by its dry weight, turbidity (optical density), plate counts etc. Whereas, indirect methods of measuring cell density are done by measuring the concentration of proteins, ATP or DNA content. Batch growth kinetics of a microbe follows a growth curve with lag phase as the initial phase during which cells adapt to a new environment. Multiple lag phases occur if the media is supplemented with more than one sugar and such type of growth is referred to as diauxic growth. Following the lag phase is the log phase in which the cell mass and cell number increases exponentially and then the depletion of nutrients starts which indicates the deceleration phase. The accumulation of toxic products results in deceleration phase after which stationary phase commences in which growth rate equals the death rate. The continuous growth kinetics accessed by a perpetual feeding process in which the growth is controlled by the concentration of the rate limiting nutrient.

Microbial growth kinetics explains the relationship between the specific growth rate of a microbe and its substrate concentration. Microbial growth kinetics largely depends on the laboratory culture conditions. In batch culture, microbial cell composition and its state change as a function of time and thus the rate of increase in biomass concentration was monitored. Alternatively, in continuous culture the concentration of substrate is at equilibrium and the culture grows at stable physiological state which provides more precise and reproducible data. However, the constant growth conditions represent an artificial growth environment which does not explain many microbial kinetic phenomena. Thus, growth of microbial cells was performed under mixed substrates rather than single substrate to understand the growth kinetics of microorganisms in their natural environment. The substrate such as nutrients (carbon and nitrogen sources), hormones and growth factors influence the growth pattern of microbial and mammalian cells. Substrate limited and substrate-sufficient growth would be observed on the basis of the relative availability of the substrate and the organisms utilize more substrate and energy under substrate sufficient conditions which in turn produces different patterns of product formation. A term describing the residual substrate concentration in Leudeking-Piret model was thus extended in the product formation kinetics.

Product of interest is traditionally achieved in the fermentation industry by metabolic engineering of few microorganisms which involves many genetic engineering techniques. The complexity of such genetic modifications and microbial metabolism due to various interconnected pathways urges the need to focus on developing mathematical models for identifying targets of metabolic engineering.

Mathematical models are kinetic models which explain the relationship between rates and the concentration of reactants/products and allows to predict the rate of conversion of reactions in to products. This simulated model thus paved way for the optimal design of the operating conditions and operating design of the process for optimal product formation. Qualitative models were mostly used by researchers rather than quantitative models for gene expression systems as quantitative prediction of process parameters are complicated. However, with the advancements in experimental techniques of life sciences and using powerful computer technology, complex mathematical models were developed which is used for the design of various bioprocesses. Industrial Biotechnology largely makes use of such mathematical models and saves time and resources with a clear understanding of strategies to optimize the product yield. Other potential uses of mathematical models include increasing the range of substrates, reduction of undesirable product formation and on the whole optimization of fermentation processes .

Studying growth of a microorganism is the basis of biotechnological exploitation of microflora for production of desired product. Optimization of growth of microorganism in a particular media is desirable due to economical and availability of particular growth constituent in a region. Despite this, some microorganisms have specific requirement and they grow in a particular growth media. The presented overview thus provides a knowledge on the fundamental basics of microbial growth kinetics and energetics which forms the basis for bio-engineering in optimizing, producing and purification of commercially novel products.

2. Growth kinetics

Classified based on the relationship between product synthesis and energy generation in the cell:

- Growth associated
- Non-growth associated
- Mixed-growth associated

2.1 Growth associated

Growth linked products are formed by growing cells and hence primary metabolites. Figure 1 clearly shows that product is formed simultaneously with growth of cells. That is product concentration increases with cell concentration. The formation of growth associated product may be described by Eq. (1);

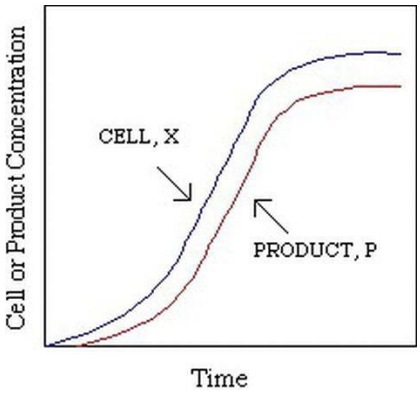


Figure 1.

Growth associated.

$$dPdt=rp=qpXdPdt=rp=qpXE1$$

where P = concentration of product

qp = specific rate of product formation

X = biomass concentration.

2.2 Non-growth associated

They are formed by cells which are not metabolically active and hence are called secondary metabolites. Figure 2 clearly shows that product formation is unrelated to growth rate but is a function of cell concentration. The formation of Non-growth associated product may be described by Eq. (2);

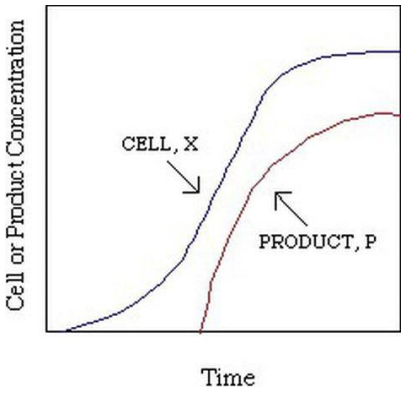


Figure 2.

Non-growth associated.

qp=β=constantqp=β=constantE2

2.3 Mixed-growth associated

The product formation from the microorganism depends on both growth and Non-growth associated. It takes place during growth and stationary phases. In Figure 3, product formation is a combination of growth rate and cell concentration. The formation of Mixed-growth associated product may be described by Eq. (3);

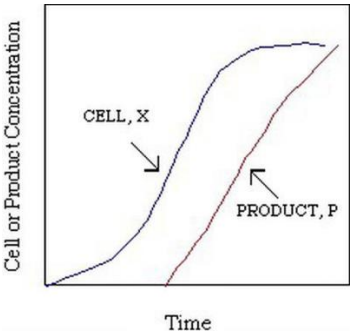


Figure 3.

Mixed growth associated.

qp=αμ+βqp=αμ+βE3

2.4 Production kinetics

Microbial growth kinetics, i.e., the relationship between the specific growth rate (μ) of a microbial population and the substrate concentration (s), is an indispensable tool in all fields of microbiology, be it physiology, genetics, ecology, or biotechnology, and therefore it is an important part of the basic teaching of microbiology. Unfortunately, the principles and definitions of growth kinetics are frequently presented as if they were firmly established in the 1940s and during the following "golden age" in the 1950s and 1960s the key publications are those of Monod. Monod, logistic, modified logistic model, and Leudeking-Piret models were used to describe the batch growth kinetics of cell. The Monod kinetic model is given as Eq. (4):

μ=μmaxSKs+Sμ=μmaxSKs+SE4

where μ is the specific growth rate (h-1), S is substrate concentration (g/L) and KS and μmax are the Monod constant (g/L) and maximum specific growth rate, (h-1) respectively.

At the end of the lag phase, the growth of microorganisms is well acclimatized for its contemporary environment. Then the cells were multiplied hastily. The major active part of the cell growth curve

which is called as the exponential (log) phase is used for the adjudication of kinetic parameters. The period of balanced growth that is the log phase, in which all components of a cell grow at the equivalent rate. Malthus model was also used for the cell growth behavior.

In Contois model, Michaelis constant is directly proportional to cell concentration and specific growth rate is inversely proportional to cell concentration which is described by Eq. (5). The Monod equation was also modified with the maintenance term which was incorporated in the Herbert model (Eq. (6)).

μ=μmaxSKsX+Sμ=μmaxSKsX+SE5

μ=(μmax+m)(SKs+S)−mμ=μmax+mSKs+S−mE6

where X is cell mass concentration (g/L) and t is time (h). Separation of variables and integrating Eq. (4) yields Eq. (5). The above equations were used to enumerate the cell growth and product accumulation during the batch experiments. The relationship between cell growth and product formation were identified by Leudeking-Piret kinetics.

Leudeking-Piret model (Eq. (7)) was used for kinetic analysis of cell production.

dpdt=αdxdt+βxdpdt=αdxdt+βxE7

where α and β are the associated and non-associated growth factor respectively. x and p show the concentration of dry cell weight (DCW) and product concentration. The Logistic equation was used to analyze the exponential growth phase kinetics while Malthus kinetics was used to express the death phase kinetics (Eqs. (8) and (9)).

dxdt=μm(1−xxm)xdxdt=μm1−xxmxE8

dxdt=μ·xdxdt=μ·xE9

x(t)=x0exp(μm·t)[1−(x0xm)(1−exp(μm·t))]xt=x0expμm·t1−x0xm1−expμm·tE10

1n(xx0)μ·t1nxx0μ·tE11

where xm, x_0 and μm are the initial DCW or biomass concentration, maximum biomass concentration and maximum specific growth rate of the microorganism, respectively. Also, tm is the required time (seed age) for maximum product concentration by the microorganism. According to Eq. (10), in order to estimate the value of the μm, a plot of 1nxxm−x1nxxm−x against t will yield a straight line that the value of its the slope corresponds to μm and the intercept equals to 1n(xmx0−1).1nxmx0−1. The substrate and product inhibitory effect on cell growth has been presented by Eq. (11), where xx is biomass concentration with respect to time and x_0 is the initial biomass concentration.

1nxxm−x=μm·t−1n(xmx0−1)1nxxm−x=μm·t−1nxmx0−1E12

The growth pattern of micro-organism followed the modified Logistic model. Maximum cell concentration was obtained for sugarcane bagasse incubated for 48 h when compared to glucose as carbon source. The experimental values deviate slightly towards the end of stationary phase because the modified logistic equation used does not distinguish the decrease in cell density that normally occurs at the end of stationary phase. Substituting Eqs. (8) and (10) into Eq. (7) and integrating, will yield Eq. (13).

p(t)=p0+αx0{exp(μm·t)[1−(x0xm)(1−exp(μm·t))]−1}+ βxmμm1n[1−(x0xm)(1−exp(μm·t))]pt=p0+αx0expμm·t1−x0xm1−expμm·t−1+ βxmμm1n1−x0xm1−expμm·tE13

Eq. (13) can be rewritten as Eq. (14)

p(t)=p0+αA(t)+βB(t)pt=p0+αAt+βBtE14

The value of dx/dt is equal to zero and $x = xm$ in the stationary phase. Using Eqs. (7) and (13), one can obtain:

β=dpdt(st·phase)xmβ=dpdtst·phasexm

The value of xm can be obtained from the experimental growth kinetic data and the value of parameter α was obtained from the slope of the linear plot of p(t)−p0−βBpt−p0−βB against $A(t)$. Eqs. (13) and (16) show the kinetic model of product production in the exponential growth phase and death phase, respectively.

p(t)=p0+αx0exp(μ·t)+βx0μexp(μ·t)pt=p0+αx0expμ·t+βx0μexpμ·tE16

=p0+αA(t)+βB(t)=p0+αAt+βBt

The resulting graph obtained from kinetic modeling of product production by Leudeking-Piret model are shown in Figure 4. It is the combination of kinetic models for better agreement between experimental data and model predictions which are employed in cell growth and Product production. The product accumulation mostly adhered to growth-associated kinetic pattern. Matlab ver. 7.12 computer software was used to define the interpretation of growth kinetic parameters.

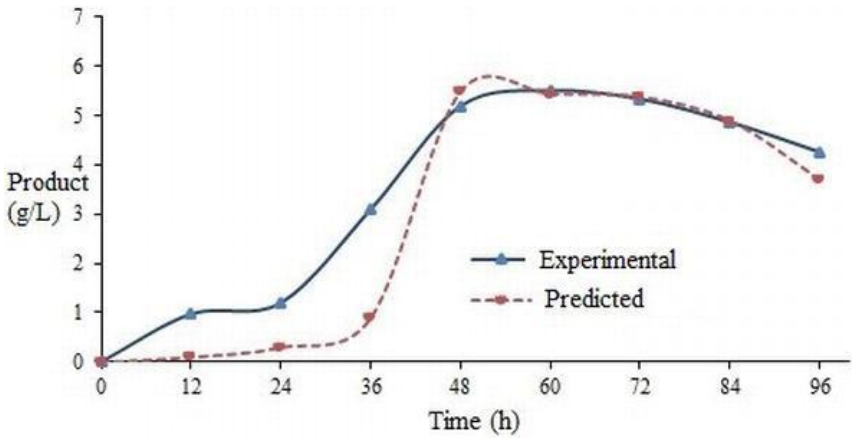


Figure 4.

The kinetic modeling of product production by Leudeking-Piret model using carbon source.

3. Conclusion

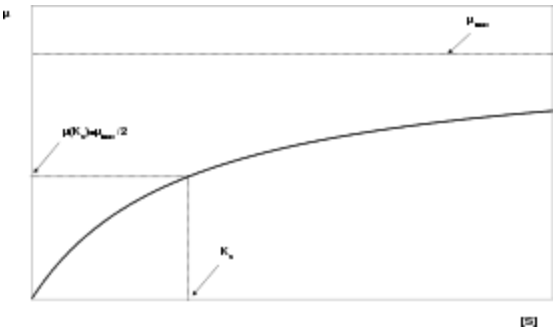
One of the very important practical applications of this model is the evaluation of the product formation kinetics. Mathematical models facilitate data analysis and provide a strategy for solving problems encountered in fermentations. Information on fermentation process kinetics is potentially valuable for the improvement of batch process performance. Finally, the product yields and substrate conversions are criteria with the main attention toward productivity.

Monod Equation

The Monod equation is a mathematical model for the growth of microorganisms. It is named for Jacques Monod (1910 – 1976, a French biochemist, Nobel Prize in Physiology or Medicine in 1965), who proposed using an equation of this form to relate microbial growth rates in an aqueous environment to the concentration of a limiting nutrient. The Monod equation has the same form as the Michaelis–Menten equation, but differs in that it is empirical while the latter is based on theoretical considerations.

The Monod equation is commonly used in environmental engineering. For example, it is used in the activated sludge model for sewage treatment.

Equation



The growth rate μ of a considered micro- organism as a function of the limiting substrate concentration [S].

The empirical Monod equation is

$$\mu = \mu_{\max} \frac{[S]}{K_s + [S]}$$

where:

- μ is the growth rate of a considered microorganism
- $\mu_{\max}$ is the maximum growth rate of this microorganism
- [S] is the concentration of the limiting substrate S for growth
- K_s is the "half-velocity constant"—the value of [S] when $\mu/\mu_{\max} = 0.5$

$\mu_{\max}$ and K_s are empirical (experimental) coefficients to the Monod equation. They will differ between microorganism species and will also depend on the ambient environmental conditions, *e.g.*, on the temperature, on the pH of the solution, and on the composition of the culture medium

Application notes

The rate of substrate utilization is related to the specific growth rate as follow

$r_{su} = -\mu X/Y$

where:

- X is the total biomass (since the specific growth rate, μ is normalized to the total biomass)
- Y is the yield coefficient

r_{su} is negative by convention.

In some applications, several terms of the form [S] / (K_s + [S]) are multiplied together where more than one nutrient or growth factor has the potential to be limiting (e.g. organic matter and oxygen are both necessary to heterotrophic bacteria). When the yield

coefficient, being the ratio of mass of microorganisms to mass of substrate utilized, becomes very large this signifies that there is deficiency of substrate available for utilization.