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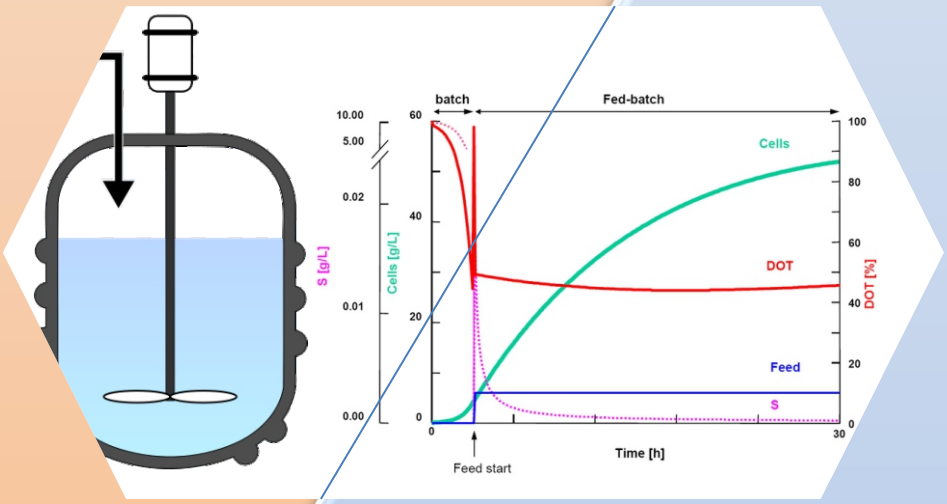
Fermentation



A COURSE BOOK

YEREVAN STATE UNIVERSITY

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FERMENTATION

A COURSE BOOK

For Students

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FERMENTATION Course Book contains information about bioprocesses, regulation and control systems of bioreactors. Growth parameters and metabolic flux of microbial cells are also included.

In this book laboratory experiments include principles of control system for bioreactors during batch fermentation and continuous fermentation. Determination of bacterial growth properties, substrate utilization and fermentation end-products generation are presented.

The course book is designed for bachelor, master and PhD students specializing in Biology and related areas.

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ABBREVIATIONS

ATP – Adenosine 5'-triphosphate
NAD – Nicotinamide adenine dinucleotide
PTS – Phosphotransferase system
PFK – Phosphofructokinase
PK – Pyruvate kinase
PDH – Pyruvate dehydrogenase
CS – Citrate synthase
PEP – Phosphoenolpyruvate
UKMs – Unstructured kinetic models
SKMs – Structured kinetic models
MCA – Metabolic control analysis
SSF – Solid state (substratum) fermentation
EIA – International Energy Administration
EIO – International Energy Outlook
Btu – British thermal units
IEA – International Energy Agency
STR – stirred-tank reactor
RTD thermowell – Resistance Temperature Detector thermowell
DO – Dissolved oxygen
ORP – Oxidation-Reduction Potential
RI detector – Refractive Index detector
DA detector – Diode Array detector
HPLC – High Performance Liquid Chromatography
GC – Gas Chromatography
CCE – Carbon Conversion Efficiency

Content

Application of fermentation in ancient times: a historical review

The process of fermentation has been widely used since ancient times. The production of beer from grist, vinegar from grain, yogurt and cheese from milk were well-established technologies in ancient Egypt. Meanwhile the mechanisms had not been discovered and scientific principles were not understood.

With the advent of the science of microbiology, and in particular fermentation microbiology, we can now shed light on these ancient and traditional activities. Many microorganisms can grow on grape sugars more readily and efficiently than yeasts, but few can withstand the osmotic pressure arising from the high sugar concentrations. Also, as sugar is fermented, alcohol concentration rises to a level at which only osmotolerant and alcohol-tolerant cells can survive. Hence, inhabitants of ancient civilizations did not need to be skilled microbiologists in order to enjoy the fruits of this popular branch of fermentation microbiology.

In fact, the scientific understanding of fermentation microbiology and, in turn, biotechnology, only began in the 1850s after **Louis Pasteur** (1822-1895) had succeeded in study of fermentation. He pioneered the study of molecular asymmetry, discovered that microorganisms cause both fermentation and disease originated the process of pasteurization, saved the beer, wine, and silk industries in France.

In 1857, Pasteur published the results of his studies and concluded that fermentation is associated with the life and structural integrity of the yeast cells rather than with their death and decay. He reiterated the view that the yeast cell is a living organism and that the fermentation process is essential for the reproduction and survival of the cell. In his paper, the words *cell* and *ferment* are used

interchangeably. The publication of this classic paper marks the birth of fermentation microbiology and biotechnology as a new scientific discipline. The process of pasteurization was named after Louis Pasteur who discovered that spoilage organisms could be inactivated in wine by applying heat at temperatures below its boiling point.

In 1878, **Wilhelm Kühne** (1837–1900) was the first to use the term *enzyme*, which is derived from the Greek word *ενζυμον* ("in leaven") to describe this process. The word enzyme was used later to refer to non-living substances such as pepsin and the word ferment used to refer to chemical activity produced by living organisms.

A second stage in the development of fermentation microbiology and biotechnology began in 1877, when **Moritz Traube** (1826-1894) proposed the theory that fermentation and other chemical reactions are catalyzed by protein-like substances and that, in his view, these substances remain unchanged at the end of the reactions. Furthermore, he described fermentation as a sequence of events in which oxygen is transferred from one part of the sugar molecule to another, culminating in the formation of a highly oxidized product (i.e., CO_2) and a highly reduced product (i.e., alcohol). Considering the limited knowledge of analytical biochemistry in general and enzymology in particular at the time, Traube's remarkable vision was to prove 50 years ahead of its time.

In 1897, **Eduard Buchner** (1860-1907), two years after Pasteur died, discovered that sucrose could be fermented to alcohol by yeast cell-free extracts and coined the term "*zymase*"

to describe the enzyme that catalyzes this conversion. The term "zymase" is derived from the Greek word "zymosis," which means fermentation. In 1907, he received the Nobel Prize in Chemistry for his biochemical research and his discovery of cell-free fermentation. In the early 1900s, the views of Pasteur were modified and extended to stress the idea that fermentation is a function of a living, but not necessarily multiplying, cell and that fermentation is not a single step but rather a chain of events, each of which is probably catalyzed by a different enzyme.

The outbreak of the First World War provided an impetus and a challenge to produce certain chemicals that, for one reason or another, could not be manufactured by conventional means. It became the basis for metabolic and biochemical engineering, as a result of which the usage of different chemical compounds led to an increase in the yield of the desired end materials.

The discoveries of *penicillin* in the late 1920s and its antibacterial properties in the early 1940s represent a landmark in the development of modern fermentation biotechnology. Once the antibacterial spectrum of penicillin was determined and found to be far from universal, pharmaceutical companies began the search for other substances with antibacterial activity. These screening programs led to the discovery of many antibacterial agents produced by various members of the actinomycetes. Although the search for new antibiotics is never over, intensive research programs involving the use of genetic and metabolic engineering were initiated with the aim of increasing the productivity and potency of current antibiotics.

Population growth around the world has led to food and fuel shortages, which forced the regulation of the fermentation process.

The next stage in the development of fermentation biotechnology was dominated by success in the use of regulatory control mechanisms for the production of *amino acids*. The first breakthrough was the discovery of glutamic acid overproduction by *Corynebacterium glutamicum* in the late 1950s and early 1960s, when a number of

Japanese researchers discovered that regulatory mutants, isolated by virtue of their ability to resist amino acid analogs, were capable of overproducing amino acids.

In the last decade, fermentation biotechnology has taken a leap forward in consequence of the rising price of oil and international concern about global warming. Brazil has been a pioneer since the 1970s in developing *bioethanol* production from its huge sugarcane industry and alongside it an expanding industry for production of ethanol-utilizing cars; as a result, today about 80% of Brazilian vehicles are fueled by>20% bioethanol–gasoline mixtures. The production of bioethanol or the capacity to make it appears to have a buffering capacity against further increases in oil prices. There are many situations in which bioethanol production represents a logical alternative to farming subsidies, such as wheat production in the European Union. There is, however, valid opposition to "first generation" bioethanol as a sustainable biofuel on grounds of minimal reduction of global warming and competition with food supply. Fortunately, fermentation biotechnology has an answer. Agricultural residues such as corn stover, cereal straws, and palm-oil wastes are lignocellulosic biomass that could dwarf current bioethanol production. Harvested factory residues, such as sugarcane bagasse, corn cobs, wheat bran, palm oilcake, and so on represent an immediate opportunity. Lignocellulosic residues alone could yield sufficient bioethanol to fuel all the cars in the world.

Geobacilli can be engineered to produce ethanol from hemicellulosic sugars with yields equivalent to those from yeast fermentations of starch sugars. They have the additional advantages of extremely rapid continuous fermentations at high temperatures in which ethanol vapor can be removed continuously from the broth. Hence, although such fermentations have not yet been commercialized, calculations indicate that the production cost will be well below that of cane bioethanol or gasoline, so the scene is set for the evolution of *biorefineries*.

The use of whole-cell biocatalysts for the large-scale production of biomolecules, biopharmaceuticals, fine chemicals, and biofuels has recently been recognized as an urgent need by industrialists and academics alike. Many studies are focused on increasing the efficiency of primary feedstock conversion to desirable end product and improving the robustness of *wholecell biocatalysts*. The new innovations in functional genomics, proteomics, metabolomics, bio-informatics, systems biology, and more recently in the use of whole-cell biocatalyst in microbial-cell factories will undoubtedly play a major role in transforming our world in an unparalleled way, despite political, ethical, and cultural differences.

Fermentation in industry: modern approaches

Microbial fermentations are currently used for the production of a diverse array of biomolecules including amino acids, fine chemicals, solvents, enzymes, hormones, and antibiotics. Such diversity may be attributed to many factors, including the high surface-to-volume ratio and the ability to utilize a wide spectrum of carbon and nitrogen sources. Besides, the nutrients required for growth also include trace elements, such as phosphate, potassium, sulfur, irons, and magnesium. The necessity for such a multitude of inputs is paramount as it is required for enzymic activities.

The conversion of substrates and simple salts to biomass occurs via four stages:

1. The conversion of substrate (glucose) and inorganic phosphate (P_i) into a whole host of biosynthetic precursors together with reducing powers and ATP.
2. Biosynthesis of monomers, the conversion of biosynthetic monomers (amino acids, nucleotides, sugars, and fatty acids). This stage is carried out through the activities of various enzymes of intermediary metabolism and requires the presence of nitrogen (NH_4) and sulfate (SO_4).
3. Polymerization of monomers into polymers, so that amino acids are converted into protein, while nucleotides are converted to RNA and DNA, and so on.
4. Assembly of polymers into various components of biomass, e.g., nucleoid, inclusion bodies and cell membranes.

Central metabolism in *Escherichia coli*

Microorganisms are capable of detecting extracellular and intracellular metabolites by virtue of their ability to sense and transmit signals to sensory control mechanisms, e.g., two-component transduction mechanisms and transcription factors. Activated transcription factors, in turn, modify (activation or deactivation) the **make** up of the enzymic machinery of central metabolism in such a way that ensures uninterrupted flow of glycolytic intermediates into central metabolism. It follows any given change in the metabolic environment that leads to a change in the threshold concentrations of signal metabolites will be sensed and transmitted to a specific transcription factor, which in turn activates or deactivates (suppress) the expression of certain genes.

Escherichia coli is a facultative anaerobic, Gram-negative organism and capable of using a wide spectrum of organic carbon sources for heterotrophic growth. The availability of electron acceptors triggers the strategies used for energy production – respiration or fermentation. In most studies that describe the use of *E. coli* for applied processes, sugars are used as carbon and electron source. Their import can be achieved via different uptake machineries. Glucose, for example, is mainly imported and simultaneously phosphorylated to glucose-6-phosphate using the phosphotransferase system (Fig. 1). Nevertheless, it has been shown that the galactose and mannose uptake machineries can also translocate glucose, which then enters the cytoplasm in its unphosphorylated form and is converted to glucose-6-phosphate by glucokinase. The glycolysis pathway processes the phosphorylated sugar into two molecules of pyruvate, which is accompanied by the release of two ATP and two NADH molecules. Under oxic conditions, pyruvate is converted to acetyl-CoA and carbon dioxide by the pyruvate dehydrogenase complex. This multimeric enzyme is downregulated under anaerobic conditions and is controlled by the $NADH/NAD^+$ ratio as well as the pyruvate

concentration. Under oxic conditions, acetyl-CoA is further processed within the citric acid cycle. This gives rise to the production of more ATP and reducing equivalents.

Escherichia coli is also able to respire under anoxic conditions, and can use a variety of substances in the absence of oxygen as electron acceptors. Nevertheless, the absence of oxygen triggers a downregulation of the citric acid cycle, which leads to an incomplete oxidation of sugars. Under these conditions, acetate is formed as the main product. Pyruvate dehydrogenase is replaced by pyruvate formate lyase, which prevents the release of NADH during pyruvate consumption and instead catalyzes the formation of formate and acetyl-CoA. The latter is converted to the accumulating acetate by the activity of phosphotransacetylase (pta) and acetate kinase (ack). The two central regulators fumaratenitrate-reduction (FNR) and ArcAB (anoxic redox control) mediate the distinction between oxic and anoxic metabolism.

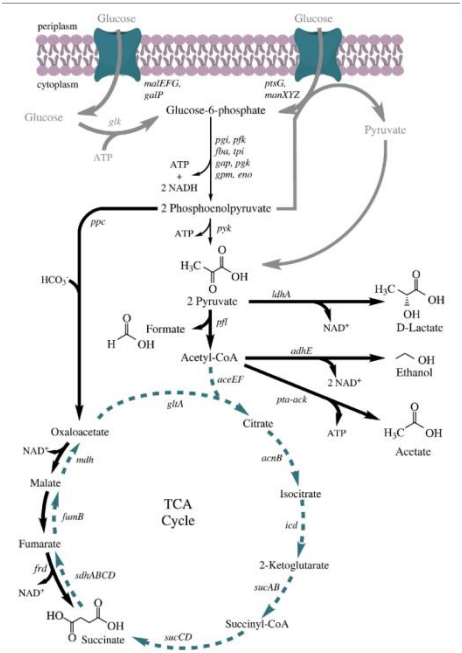

Figure 1. Anaerobic fermentative metabolism in Escherichia coli. Chemical structures are shown for all mixed-acid fermentation products and pyruvic acid. Bold gray arrows: glucose transport systems; thin black arrows: glycolysis; bold black arrows: fermentative reactions; dashed, green arrows: TCA cycle, only anabolic functions, completely active under oxic conditions. Genes: malEFG (maltose ABC transporter), galP (galactose:H+ symporter), ptsG (fused glucose-specific PTS enzyme: IIB and IIC component), manXYZ (mannose PTS permease), glk (glucokinase), pgi (glucose-6-phosphate isomerase), pfk (6-phosphofructokinase), fba (fructose-bisphosphate aldolase), tpi (triosephosphate isomerase), gap (glyceraldehyde 3-phosphate dehydrogenase), pgk (phosphoglycerate kinase), gpm (phosphoglycerate mutase), eno (enolase), pyk (pyruvate kinase), ppc (phosphoenolpyruvate carboxylase), ldhA (lactate dehydrogenase), pfl (pyruvate formate lyase), aceEF (pyruvate dehydrogenase complex), adhE (alcohol dehydrogenase), pta (phosphate acetyltransferase), ack (acetate kinase), gltA (citrate synthase), acnB (aconitase), icd (isocitrate dehydrogenase), sucA (2-oxoglutarate decarboxylase), sucB (2-oxoglutarate dehydrogenase), sucCD (succinyl-CoA synthetase), sdhABCD (succinate dehydrogenase), fumB (fumarate hydratase), frd (fumarate reductase), and mdh (malate dehydrogenase).

Under fermentative conditions, a mixture of succinate, formate, acetate, lactate, and ethanol is produced to maintain redox balance. Ethanol formation is established using alcohol dehydrogenase (adhE), which catalyzes the reaction from acetyl-CoA to ethanol with the consumption of two NADH molecules. The production of lactate is catalyzed by the soluble lactate dehydrogenase (ldhA) via reduction of pyruvate (consumption of one NADH molecule).

Succinate formation starts with the carboxylation of phosphoenolpyruvate to oxaloacetate by PEP-carboxylase (ppc), and is subsequently achieved via the activity of malate dehydrogenase (mdh), fumarase (fumB), and fumarate reductase (frd).

∝-ketoglutarate plays a central role in carbon and nitrogen metabolism not only through its ability to provide a carbon skeleton for the glutamate and glutamine family of amino acids, but also for its ability to act as a signal metabolite, which allows the organism to better adapt to changes in the availability of nutrients. It follows that *E. coli* is capable of sensing changes in the intracellular concentration of ∝-ketoglutarate and responding through the operation of signal transduction systems that monitors the prevailing nutritional status and generates the appropriate metabolic response.

During growth of *E. coli* under nitrogen limitation, the conversion of ∝-ketoglutarate to glutamate ceases, thus leading to the accumulation of ∝-ketoglutarate. Should the intracellular concentration of ∝-ketoglutarate rise above a certain threshold, the organism detects such a rise as a signal and responds by feedback inhibiting the activity of enzyme 1 of the PTS, thus restricting the flow of intermediates into the glycolytic pathway until an adequate supply of nitrogen source is made available. Directionality of glycolytic intermediates into central metabolism is assured by the operations of irreversible reactions, catalyzed by PFK, PK, PDH, and CS, as well as efficient allosteric control mechanisms (feed forward activation of PK and feedback inhibition of PFK by Fructose 1, 6-bisphosphate and PEP, respectively) that fine control the flow of intermediates at crucial junctions.

Regulation of metabolic pathways

Primary metabolites are microbial products made during the exponential phase of growth whose synthesis is an integral part of the normal growth process. They include intermediates and end products of anabolic pathways leading to the formation of monomers, which are used by the cell as building blocks (e.g., amino acids and nucleotides) for the biosynthesis of polymers (e.g., protein and DNA) and coenzymes (e.g., vitamins). On the other hand, primary metabolites of catabolic pathways (e.g., citric acid, acetic acid, and ethanol) are not biosynthetic precursors but are essential for growth as they are related to energy generation, redox balance, and substrate utilizations. Industrially, the most important primary metabolites are amino acids, nucleotides, vitamins, solvents, and organic acids.

Microbial metabolism is a conservative process that usually does not expend energy or nutrients to make compounds already available in the environment and does not overproduce components of intermediary metabolism. Coordination of metabolic functions ensures that, at any given moment, only the necessary enzymes, and the correct amounts of each, are made. Once a sufficient quantity of a given metabolite or precursor is made, either the synthesis of the enzymes concerned is turned "off" at the level of transcription (*repression*) or their activities are turned down (*inhibition*) through a number of specific regulatory control mechanisms such as feedback inhibition and covalent modifications.

While the majority of anabolic enzymes are subject to repression, most catabolic enzymes are subject to induction. The latter is a control mechanism by which a substrate (or a compound structurally similar to the substrate) "turns on" the synthesis of the enzymes required for its uptake and initiation of metabolism. Enzymes that are synthesized as a result of genes being turned "on" in response to signal molecules (substrates) are called **inducible enzymes**, with the substance that activates gene transcription being referred to as the inducer. Inducible

enzymes are produced only in response to the presence of their substrate or substrate analogs, in other words, they are produced only when needed.

Catabolite inhibition and catabolite repression mechanisms allow microorganisms to preferentially utilize one substrate in preference to another, thus facilitating faster growth through conserving energy and biosynthetic precursors. For example, in the presence of lactose and glucose, *Escherichia coli* preferentially utilizes glucose and prevents the uptake of lactose through catabolite inhibition mechanism. *E. coli* also switches off the transcription of the *lac* operon through catabolite repression mechanism, which is also known as **carbon catabolite repression**. Such a mechanism ensures that the cell produces enzymes to metabolize the assimilated carbon source but represses the synthesis of the enzymes required for the assimilation of the competing substrate until the primary carbon substrate, in this case glucose, is fully exhausted.

Feedback regulation is predominantly used for the regulation of anabolic enzymes involved in the biosynthesis of amino acids, nucleotides, and vitamins. It exerts its function at two levels: enzyme action (feedback inhibition) and enzyme synthesis (feedback repression and attenuation). In feedback inhibition, the final metabolite of a pathway, when present in sufficient quantities, inhibits the action of the first enzyme of the pathway, thus preventing the synthesis of unwanted intermediates on the one hand and the wasting of energy on the other. Feedback repression involves the turning “off” of enzyme synthesis when the amount of the product has been made in sufficient quantities to satisfy the biosynthetic demands. The end product of the pathway acts as a co-repressor. The aporepressor specified by the regulator gene is inactive in the absence of its co-repressor and as such is unable to bind to the operator region. However, in the presence of a co-repressor, the inactive apo-repressor is converted to an active repressor that binds to the operator region, thus preventing the binding of RNA polymerase to the promoter region, which in turn brings enzyme biosynthesis to a halt.

Growth of microbial cells

The rate of product formation in a given industrial process, a significant parameter, is directly proportional to the rate of biomass formation, which is influenced directly or indirectly by a whole host of different environmental factors (e.g., oxygen supply, pH, temperature, and accumulation of inhibitory intermediates). It is, therefore, important to describe growth and production in quantitative terms.

Microbial growth is described by the following equation:

$$\frac{dx}{dt} = \frac{ax}{b} \quad (1)$$

According to this equation the rate of biomass (x) formation changes as a function of time (t) and that the rate of change is directly proportional to the concentration of a particular factor (a) such as growth substrate or temperature but is inversely proportional to the concentration of another factor (b) such as inhibitors. In Equation (1), both a and b are independent of time t, and the proportionality factor in Equation (1) can in effect be ignored.

During batch fermentation, exploration of growth curve is important profile. It is differentiated eight phases of the growth cycle (Fig. 2):

1. Lag phase
2. Acceleration phase
3. Exponential (logarithmic) phase
4. Deceleration phase
5. Stationary phase
6. Accelerated death phase
7. Exponential death phase
8. Death or survival phase

In the **lag** phase, the organism is simply faced with the challenge of adapting to the new environment. Adaptation to other carbon sources, however, may require the induction of a particular set of

enzymes that are specifically required to catalyze the transport and hydrolysis of the substrate. Irrespective of the mechanisms employed for adaptation, the net outcome at the end of the lag phase is a cell that is biochemically vibrant (i.e., capable of transforming chemicals to biomass).

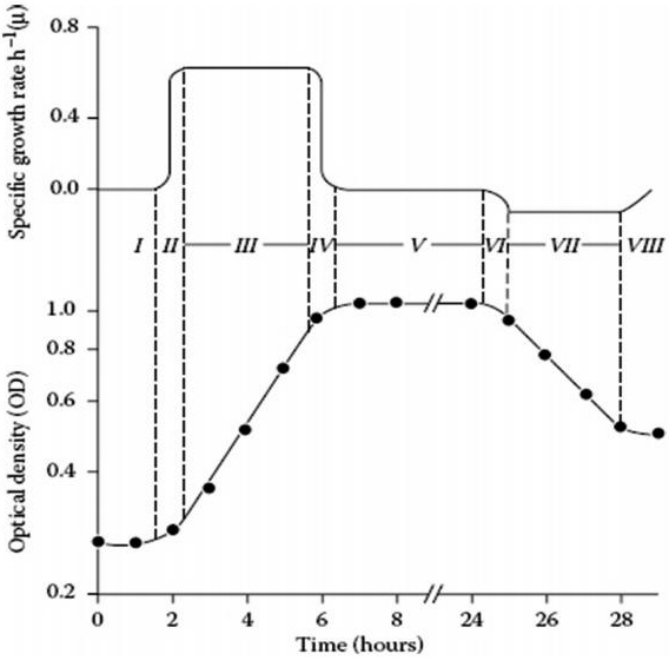

Figure 2. Typical pattern of growth cycle during the growth of microorganisms in batch cultures; the vertical dotted lines and the roman numerals indicate the changes in specific growth rate (μ) throughout the cycle.

As soon as *E. coli* adapts to the carbon source it **accelerates** into the exponential phase. Acceleration of growth en route to entry exponential growth is associated with the expression of a nucleotide binding protein, the level of which increases as a function of *growth rate (μ)* and peaks during the log phase.

In the **exponential phase**, each cell increases in size, and providing that conditions are favorable, it divides into two, which, in

turn, grow and divide; and the cycle continues. During this phase, the cells are capable of transforming the primary carbon source into biosynthetic precursors, reducing power and energy, which is generally trapped in the form of ATP, PEP, and proton gradients. The biosynthetic precursors thus generated are then channeled through various biosynthetic pathways for the biosynthesis of various monomers (amino acids, nucleotides, fatty acids, and sugars) that, in turn, are polymerized to give the required polymers (proteins, nucleic acids, ribonucleic acids, and lipids). Finally, these polymers are assembled in a precise way, and the cell divides to give the new biomass characteristic of each organism. The time span of each cycle (cell division) is known as generation time or doubling time, but because we generally deal with many millions of cells in bacterial cultures, the term *mean generation time (T)* is more widely used to reflect the average generation times of all cells in the culture.

The relationship between the mean generation time (T) and the specific growth rate (μ) can be described mathematically by

$$\ln 2 = \mu T, \text{ or } \mu = \frac{ln2}{T}$$

Because ln2=0.693, then

$$T = \frac{0.693}{\mu}$$

During this phase, all bacterial cells are identical and that the doubling time is constant with no loss in cell numbers due to cell death. As the exponential phase draws to an end, the organism enters the **stationary phase** and then the **death phase**.

Growth of microbial cells is the result of many chemical reactions: fueling reactions that converts nutrients into a set of 12 precursor metabolites, biosynthetic reactions that converts these 12 precursor metabolites into building blocks like amino acids, nucleotides, and fatty acids, and assembly reactions that polymerize building blocks into proteins, DNA, complex lipids, and so on. The growth occurs in a constant volume of medium with one growth-

limiting substrate component that is used by the unicellular organism (either a bacterium or yeast). Cell growth is generally quantified by specific growth rate μ (h^{-1}), which for such a culture is given by

$$\mu = \frac{1}{x}\frac{dx}{dt}$$

where x is the biomass concentration (or cell number). The specific growth rate is related to the *doubling* time t_d (h) of the biomass through

$$t_d = \frac{\ln 2}{\mu}$$

The doubling time td is equal to the generation time for a cell (i.e., the length of a cell cycle for unicellular organisms), which is frequently used by life scientists to quantify the rate of cell growth.

Fermentation balance

Apart from the fraction of carbon used for biomass formation, the remainder is partitioned between products and by-products, including carbon dioxide. The ratio of recovered carbon to that present at the onset of fermentation is referred to as the **carbon balance or carbon recovery index**. Such a balance or an index is a measure of efficiency: the higher the index, the higher the carbon recovery, and the more efficient the fermentation process. The carbon balance is generally calculated by working out the number of moles (or mill moles) produced of a given product per 100 moles (or mill moles) of substrate utilized. The number of carbon atoms in each respective molecule can then be multiplied by the value obtained. The resulting values for the products in question can then be totaled and compared with that of the substrate. If the values are equal (i.e., a 1:1 ratio), then a complete recovery of carbon into product formation has been achieved. Although this is theoretically possible, our experience indicates otherwise, as part of this carbon is used for maintenance and assimilation to support growth, however slow. A complete carbon balance can, therefore, be calculated for any given fermentation if the fraction of carbon diverted toward biosynthesis and maintenance is determined.

In addition to the carbon balance, some fermentations demand calculation of the **redox balance**. Fermentations of sugars and other primary carbon sources give rise to a whole host of different intermediates; some of which are phosphorylated (energy-rich), while others are not. While biosynthetic intermediates are utilized for the biosynthesis of monomers, other intermediates are produced in excess and this is balanced by their excretion into the medium to redress the redox balance within the cell, as is the case with alcohol excretion or to generate ATP, as is the case with acetate excretion or to regenerate the proton motive force, as is the case with lactate excretion. The stoichiometry of product and by-product formations of any given

fermentation process can be ascertained by carefully analyzing the culture filtrates at different stages. From a physiological standpoint and in order for fermentation to go to completion, the redox balance must be maintained.

Kinetic models for fermentation

Kinetic modeling expresses verbally or mathematically correlations between rates and reactant or product concentrations, permit a prediction of the degree of conversion of substrates and the yield of individual products at other operating conditions. If the rate expressions are correctly set-up, it may be possible to express the course of an entire fermentation experiment based on initial values for the components of the state vector (e.g., concentration of substrates). This leads to simulations, which may finally result in an optimal design of the equipment or an optimal mode of operation for a given system. The basis of kinetic modeling is to express functional relationships between the forward reaction rates of the reactions considered in the model and the concentrations of the substrates, metabolic products, biomass constituents, intracellular metabolites, and/or biomass.

An important characteristic of modeling is the assumption of homogeneous or heterogeneous conditions. In this sense, a homogeneous system is related to a single continuous phase. In most cases, bioreactors are described as single liquid phases. However, if the biofilm is included in the study, a solid or semisolid phase needs to be considered in the model. On the other hand, heterogeneous systems are related to the description of two or more continuous phases and the interactions between them. Complex heterogeneous systems can be described as multiple phases: liquid, solid or semisolid, and gaseous phases (e.g. solid-state fermentation).

Unstructured kinetic models (UKMs) represent, in a simple global point of view, the metabolic behavior of the biomass cell production. Mainly, mathematical descriptions for microbial growth kinetics in fermentation processes are based on semiempirical observations. From simple experimental data, we can obtain information to represent cellular growth with unstructured kinetic models. Most of the UKMs can be divided into three terms: rate

expressions for cell growth, rate expressions for nutrient uptake, and rate expressions for metabolite production. One of the most used UKMs is Monod's model. This is one of the simplest models to deal with microbial growth, physiology, and biochemistry. The Monod equation describes the proportional relationship between the specific growth rate and low substrate concentrations (Eq. (3)).

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

where μ_{max} is the maximum specific growth rate, [S] is the substrate concentration, and K_S is the saturation constant.

In the Monod model, it is assumed that the yield of biomass from the limiting substrate is constant; in other words, there is proportionality between the specific growth rate and the specific substrate uptake rate. The disadvantage of the model is that the individual entity, regulatory complex, adaptive response to environmental changes, and capacity of cell organelles to generate various products in inherent metabolism cannot be considered.

Structured kinetic models (SKMs) are in one sense improvements to the unstructured models because some basic mechanisms of the cellular behavior are at least qualitatively incorporated. These models describe changes in cell population. The liquid phase (abiotic phase) usually contains nutrients for cell growth and some extracellular metabolites. The microorganisms suspended in the liquid phase behave as multicomponent systems. SKMs consider the internal structure of cells (e.g. mitochondria), and the description of cell growth and its metabolism is used to assume a more accurate growth rate. The information used is a starting point to generate schemes that represent more accurately the growth of microorganisms and their cellular components. The complexity of the information variables and parameters increases in SKMs with the mathematical representation of cellular growth.

Single-cell models are in principle an extension of the compartment models, but with the description of many different

cellular functions. Furthermore, these models depart from the description of a population and focus on the description of single cells. This allows consideration of characteristic features of the cell and it is therefore possible to study different aspects of cell function:

- Cell geometry can be accounted for explicitly, and so it is possible to examine its potential effects on nutrient transport.
- Temporal events during the cell cycle can be included in the model, and the effect of these events on the overall cell growth can be studied.
- Spatial arrangements of intracellular events can be considered, even though this would lead to significant model complexity.

To set up single-cell models, it is necessary to have a detailed knowledge of the cell, and single-cell models have therefore only been described for well-studied cellular systems.

Despite the level of detail, the single-cell models are normally based on an empirical description of different cellular events (e.g., gene transcription and translation). This is a necessity because the complexity of the model would become very high if all these individual events were to be described with detailed models that include mechanistic information. In many cases, however, it is interesting to study these events separately, and for models where mechanistic information is included, they have to be used.

Molecular mechanistic models are normally set up at the molecular level. Many different models of this type can be found in the literature, but most fall in one of two categories: *Gene transcription models* and *Pathway models.* Gene transcription models aim at quantifying gene transcription based on knowledge of the promoter function. In pathway models the individual enzymatic reactions of a given pathway are described with enzyme kinetic models, and it is therefore possible to simulate the metabolite pool levels and the fluxes through different branches of the pathway.

The net result of the many biochemical reactions within a single cell is the conversion of substrates to biomass and metabolic end

products. Different types of cellular metabolic reactions are determined:

Assembly reactions carry out chemical modifications of macromolecules, their transport to prespecified locations in the cell, and, finally, their assembly to form cellular structures such as cell walls, membranes, the nucleus, and so on.

Polymerization reactions represent directed, sequential linkage of activated molecules into long (branched or unbranched) polymeric chains. These reactions lead to the formation of macromolecules from a set of building blocks such as amino acids, nucleotides, and fatty acids.

Biosynthetic reactions produce the building blocks used in the polymerization reactions. They also produce coenzymes and related metabolic factors, including signal molecules. Furthermore, a large number of biosynthetic reactions occur in functional units called biosynthetic pathways, each of which consists of sequential reactions leading to the synthesis of one or more building blocks.

Fueling reactions produce the metabolites needed for biosynthesis. Additionally, they generate Gibbs free energy in the form of adenosine-5’-triphosphate (ATP), which is used for biosynthesis, polymerization, and assembling reactions. Finally, the fueling reactions produce the reducing power needed for biosynthesis. The fueling reactions include all biochemical pathways referred to as catabolic pathways (degrading and oxidizing substrates).

Key points for metabolic flux control

Industrial biotechnologists are generally, but not entirely, of the view that flux through a given pathway is usually limited by one step. Such a step is termed the rate-limiting step or the bottleneck with the enzyme catalyzing such a step being referred to as the "pacemaker." Although several approaches have been used as an alternative to the rate-limiting step, it is Kacser's theory of **metabolic control analysis** (MCA) that has grown in stature since its inception in 1973 and proved, without undermining the intellectual capacity of other approaches, to be the ultimate approach. The controversy over the question of whether a given method can be used successfully to predict or determine the relative contribution of each enzyme to the overall flux in a given pathway was finally resolved when Kacser and Burns (1973) and Heinrich and Rapaport (1974) independently proposed the theory of MCA. The fundamental difference between the rate-limiting step as a concept and that of the theory of MCA is that whereas the former is a qualitative parameter, the latter examines biological systems in a quantitative way that excludes bias, expectations, or preconceived ideas.

It is proposed that qualitative terms used to describe an enzyme as "rate-limiting," "bottleneck," or "pacemaker" should be abandoned and replaced by the term "**rate-controlling**" and further qualified by the flux control coefficient to describe, in quantitative terms, the capacity of the enzyme in question with respect to flux control within a given pathway.

The **flux control coefficient** of a particular enzyme is a system property (i.e., its value is not entirely independent of other enzymes in the pathway). The interrelationship between the flux control coefficients in a given pathway is governed by the *summation theorem*, which dictates that the total sum of all flux control coefficients adds up to 1.

In a steady state, the influence of a particular metabolite on flux through a given enzyme on the one hand and the whole pathway on the other can be determined from the **enzyme's elasticity coefficient**, a quantitative term that is directly related to the kinetic properties of the enzyme involved.

The metabolic interrelationship between the flux control coefficient and the elasticity coefficient is described by the *connectivity theorem*, which takes into account the kinetic properties of each of the enzymes involved.

The action of external effectors on metabolic flux can be assessed by measuring the **response coefficient**, which, to a large extent, is dependent on the flux control coefficient and the elasticity coefficient of the enzyme with respect to the effector. For an effector to be able to influence the flux through a certain enzyme, the values of the aforementioned coefficients must be relatively high.

Activating or increasing the catalytic activity of a single enzyme is not usually accompanied by a significant increase in flux (productivity), even with enzymes possessing a relatively large flux control coefficient. This is simply because the flux control shifts to other enzymes as the target enzyme is activated. This, in turn, implies that amplification of single enzymic activity is not a viable option for increasing productivity. However, this limitation does not apply to the reduction of catalytic activity because reduction or inactivation is generally accompanied by a considerable drop in flux.

A **universal method** has been developed to calculate the exact degree of overexpression required for increasing flux to product formation by a certain factor without perturbing the steady-state fluxes in other enzymes.

Increasing the concentration of an **effector** that activates all enzymes in the pathway will be accompanied by an appreciable increase in flux and, in turn, yield. Furthermore, an effector that stimulates the activity of more than one enzyme in a given pathway

may lead to increase in flux (productivity), particularly if those enzymes share a relatively high flux control coefficient.

Evasion and **subversion** are the most suitable strategies for increasing fluxes to product formation without adversely affecting the intracellular concentrations of metabolites; although evasion has the added advantage of being applicable to all metabolic pathways because it does not make any assumption about the regulatory control systems used in vivo.

However, subversion affords a practical strategy because it only involves the manipulation of one enzymic activity and as such may prove more of an attractive proposition for industrialists. Subverting feedback inhibition by the desired end product can simply be achieved by generating a mutant that leaks the end product into the medium. Such a system is highly desirable because the recovery and downstream processing become more effective and less expensive.

Types of fermentation

Eight main types of fermentations are described:

Batch fermentation is a closed culture system, because initial and limited amount of sterilized nutrient medium is introduced into the fermenter (Fig. 3). The medium is inoculated with a suitable microorganism and incubated for a definite period for fermentation to proceed under optimal physiological conditions. Oxygen in the form of air, an antifoam agent and acid or base, to control the pH, are being added during the course of fermentation process. During the course of incubation, the cells of the microorganism undergo multiplication and pass through different phases of growth and metabolism due to which there will be change in the composition of culture medium, the biomass and metabolites. The fermentation is run for a definite period or until the nutrients are exhausted. The culture broth is harvested and the product is separated.

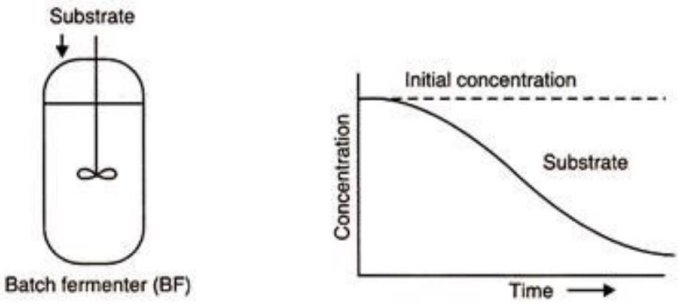

Figure 3. Batch fermenter

Batch fermentation may be used to produce biomass, primary metabolites and secondary metabolites under cultural conditions supporting the fastest growth rate and maximum growth would be used for biomass production. The exponential phase of growth should be prolonged to get optimum yield of primary metabolite, while it should be reduced to get optimum yield of secondary metabolites. The

used medium along with cells of microorganism and the product is drawn out from the fermenter. When the desired product is formed in optimum quantities, the product is separated from the microorganism and purified later on.

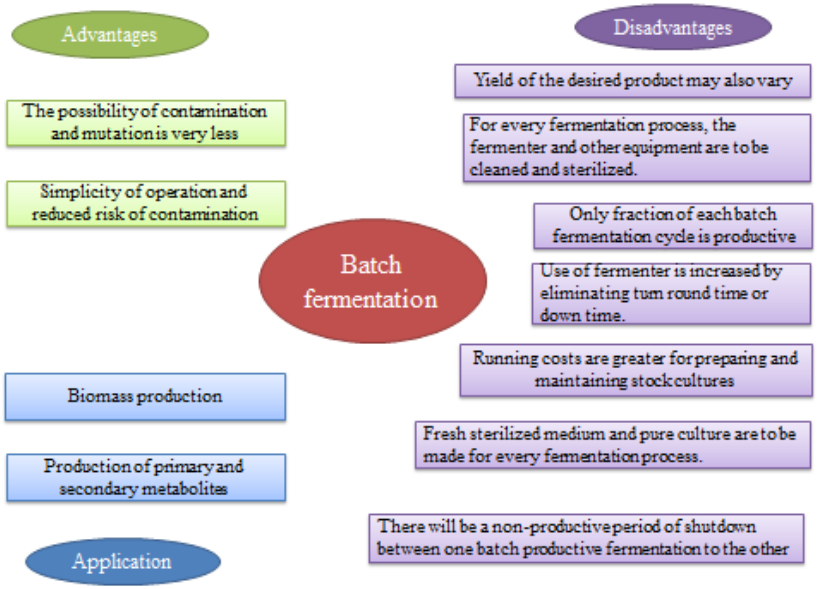

Figure 4. Advantages, disadvantages and applications of Batch fermentation

Continuous fermentation is a closed system of fermentation, run for indefinite period. In this method, fresh nutrient medium is added continuously or intermittently to the fermenter and equivalent amount of used medium with microorganisms is withdrawn continuously or intermittently for the recovery of cells or fermentation products (Fig. 5). As a result, volume of the medium and concentration of nutrients at optimum level are being maintained. This has been operated in an automatic manner. The continuous fermenter has its maximum use that takes long time to reach high productivity,

reduces down time and lowers the operating costs. In continuous mode, starting medium and inoculum are added to the fermenter.

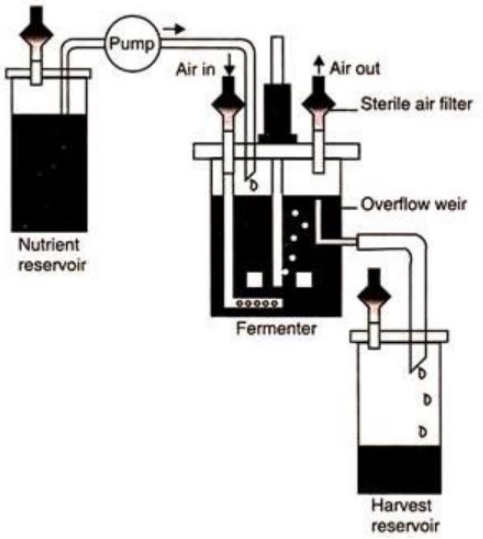

Figure 5. Continuous fermenter

After the culture is grown the fermenter is fed with nutrients and broth is withdrawn at the same rate maintaining a constant volume of broth in the fermenter. In continuous mode with cell cycle, the cell mass is returned to the fermenter using micro filtrations with bacteria or screens with fungal mycelium.

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

1. Single Stage Fermentation: In this process, a single fermenter is inoculated and the nutrient medium and culture are kept in continuous operation by balancing the input and output of nutrient medium and harvested culture, respectively.
2. Recycle Fermentation: In this method, a portion of the medium is withdrawn and added to the culture vessel. Thus, the culture is recycled to the fermentation vessel. This method

is generally adopted in the hydrocarbon fermentation process. The recycling of cells provides a higher population of cells in the fermenter which results in greater productivity of the desired product.

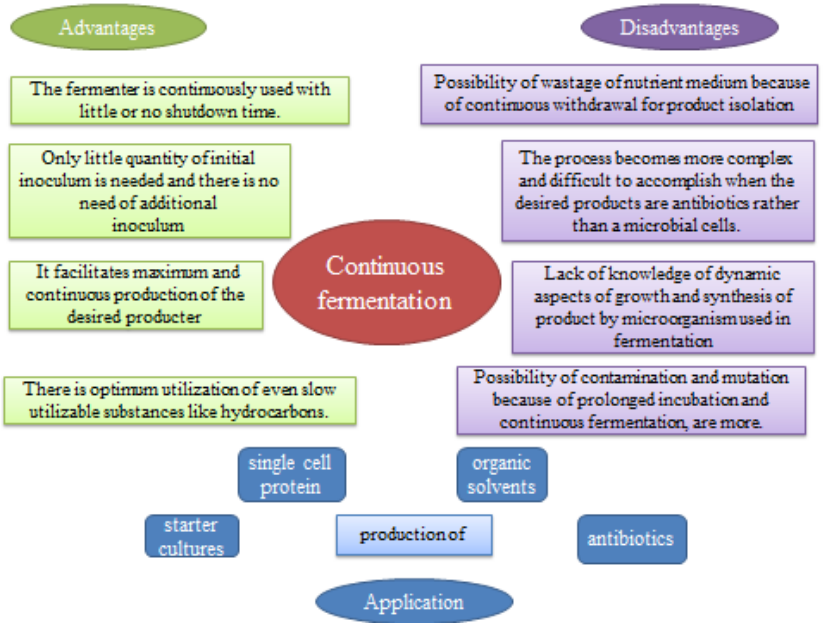

Figure 6. Advantages, disadvantages and applications of Continous fermentation

3. Multiple Stage Fermentation: In this process, two or more fermenters are employed simultaneously and the fermentation is operated in a sequence. Different phases of fermentation process like growth phase and synthetic phase are carried out in different fermenters. Generally, growth phase is allowed in the first fermenter, synthetic phase in the second and subsequent fermenters. This process is adapted particularly to those fermentations in which growth and synthetic activities of the microorganisms are not simultaneous. Synthesis is not

growth related but occurs when cell multiplication rate has slowed down.

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

Turbidostat Method: In this method the total cell content is kept constant by measuring the culture turbidity at a regular interval of fermentation process. By turbidity measurement it is possible to the fermenter to regulate both the nutrient feed rate and the culture withdrawal rate. Fermentation, in which this method is employed, must be carried out at a low maximum cell population which leads to the usage of less amount of substrate and wastage of greater amount of substrate as unused and residual medium, which is removed from the fermenter along with the harvested culture.

Chemostat Method: In this method nutrient feed rate and harvest culture withdrawal rate are maintained at constant value. This is achieved by controlling the growth rate of the microorganism by adjusting the concentration of any one of the chemicals of the medium, like carbon source, nitrogen source, salts, O2 etc. which acts as a growth limiting factor. Apart from the above chemicals, sometimes the concentration of the toxic product generated in the fermentation process, the pH values and even temperature also act as growth limiting factors. This method is employed more often than turbidostat method because of fewer mechanical problems and presence of less amount of unused medium in the harvested culture.

Auxostat, nutristat, and pH-stat are synonymous names for a continuous culture in which the dilution rate changes to maintain a certain factor constant.

Fed batch fermentation is a modification to the batch fermentation. In this process substrate is added periodically in instalments as the fermentation progresses, due to which the substratum is always at an optimal concentration. This is essential as some secondary metabolites are subjected to catabolite repression by

high concentration of either glucose, or other carbohydrate or nitrogen compounds present in the medium.

For this reason, the critical elements of the nutrient medium are added in low amount in the beginning of the fermentation and these substrates continue to be added in small doses during the production phase. This method is generally employed for the production of substances such as penicillin. Yoshida (1973) introduced this term for the first time for feeding the substrates to the medium as the nutrients are exhausted, so as to maintain the nutrients at an optimum level.

The fed-batch fermentation may be of three types:

1. Variable Volume Fed Batch Culture: The same medium is added resulting in an increase in volume.
2. Fixed Volume Fed Batch Culture: A very concentrated solution of the limiting substrate is added at a very little amount resulting in an insignificant increase in the volume of medium.
3. Cyclic Fed Batch Culture: As it is not possible to measure the substrate concentration by following direct methods during fermentation, which is necessary for controlling the feeding process, generally indirect methods are employed. For example – in the production of organic acids, the pH value may be used to determine the rate of glucose utilization.

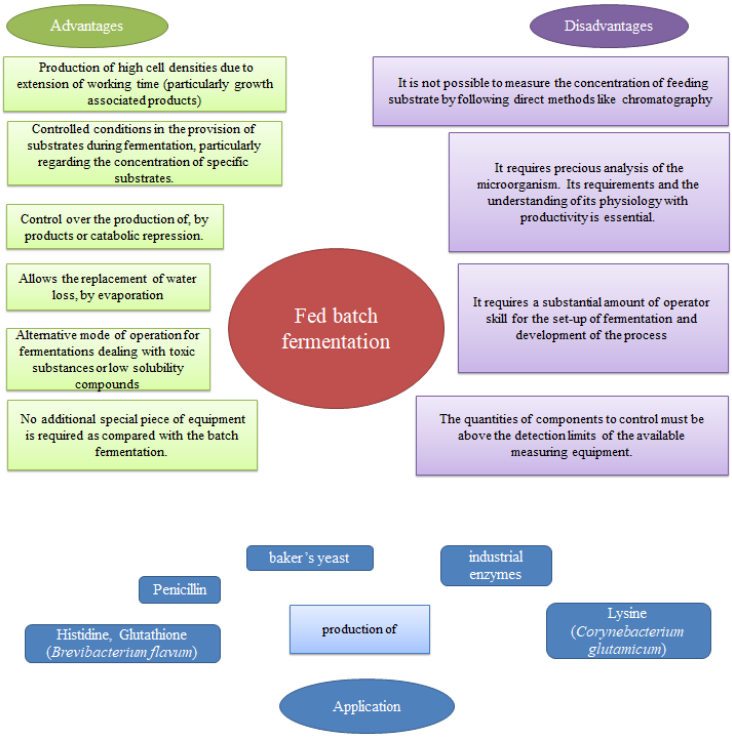

Figure 7. Advantages, disadvantages and applications of Fed batch fermentation.

A fermentation process carried out in the absence of oxygen is called as **anaerobic fermentation**. There are two types of anaerobic microorganisms, obligate anaerobic microorganisms and facultative anaerobic microorganisms. The former like *Clostridium sp*. cannot withstand oxygen or remain active only in the absence of oxygen. They remain active in the absence of oxygen and produce optimum amount of the desired product. The facultative anaerobes like lactic acid bacteria are able to withstand small amount of oxygen. However, certain organisms like yeast require an initial aeration to build up high cell yield before anaerobic conditions are created. Anaerobic conditions in the fermenter are created either by withdrawing the oxygen present in the head space by an exhaust pump and pumping

some inert gases like nitrogen, argon etc. or by flushing it out, by the emergence of certain gases like carbon dioxide or hydrogen.

A fermentation process carried out in the presence of oxygen is called as **aerobic fermentation**. In most of the commercial processes and majority of the products of human utility are produced by this type of fermentation. Fermentation can be surface culture or static and submerged.

Surface fermentations are those where the substratum may be solid or liquid. The organism grows on the substratum and draws the nutrients from the substratum. These types of fermentations are desirable where the products are based on sporulation. But it has several disadvantages such as it exposes the organism to unequal conditions, both oxygen and nutrients.

Submerged Fermentations are those in which the nutrient substratum is liquid and the organism grows inside the substratum. The culture conditions are made uniform with the help of spargers and impeller blades. Most of the industrial fermentations are of this type. The substratum which is in a liquid state and such medium is also called as broth.

Solid state (substratum) fermentation (SSF) is generally defined as the growth of the microorganism on moist solid materials in the absence or near the absence of free water. In recent years SSF has shown much promise in the development of several bioprocesses and products, SSF has been ambiguously used as solid-state fermentation or solid-substrate fermentation. However, it is proper to distinguish between two processes. Solid substrate fermentation should be used to define only those processes in which the substrate itself acts as carbon source occurring in absence or near absence of free water. On the other hand, the solid state fermentation is that fermentation which employs a natural substrate as above or an inert substrate used as solid support. Solid substrate fermentation is normally many step process involving.

For some fermentation, SSF is desirable because of following reasons:

1. In several productions, the product formation has been found superior in solid culture process.
2. The most commonly used microorganisms in the production of secondary metabolites are fungi and actinomycetes and the mycelial morphology of such organisms is ideal for their invasive growth on solid and insoluble substrates.
3. In some processes the final product is required in solid form, such as antibiotics in animal feed.
4. The capital cost of overall production process is claimed to be significantly less.
5. The fungus possess tremendous turgor pressure at the mycelial tips.
6. Microbial cells attach to solid substrate particles and completely surrounds the particle in mycelial webs.
7. It provides optimum quantity of water for growth.
8. Crude substrates can be used as the organisms can tolerate high concentration of metal ions and mineral ions.
9. Overcome catabolite repression and can be provided high substrate concentration.
10. Enzymes become extracellular otherwise intracellular in SMF. E.g.- Galactase, tannase and invertase.
11. Metabolite production phase is long.
12. Co-production of carbohydrates and proteases.
13. Enzymes produced by this will be with better properties and extra desirable components.
14. Low waste water output/less water need.
15. Reduced energy requirement.
16. Absence of foam formation.
17. Simplicity. High reproducibility.
18. Simpler fermentation media.
19. Lesser fermentation space.
20. Lower cost of downstream processing

Microbial cell factories: Importance of membrane transporters

In a typical fermentation, substrates are provided externally to the cells catalyzing the fermentation and converted to products. However, for a relative density (specific gravity) of 1, cells occupy 1 mL g^{-1} wet weight so for a fermentation that achieves even 100 mg wet cell wt mL^{-1} only approximately one tenth of the total volume is intracellular. Commonly, intracellular concentrations during the development of processes for industrial bioengineering can soon become toxic. Since thermodynamics dictates that it is standard free energies and concentrations that control the eventual outcome, it is needed to recognize, that outwith a solid substrate fermentation in which the biomass is the product the overall titer of product will be much enhanced if its internal concentrations can be decreased by secretion into the larger extracellular space. In addition, it is much easier to purify products if cells are not present.

There are different types of membrane transporter, which provide the excretion of fermentation end-products. A first distinction is whether they are equilibrative (i.e., permitting "facilitated diffusion") or whether their activities are coupled to an external free energy source such as ATP (hydrolysis) or electrochemical gradients; the latter kinds of transporter may then be concentrative in terms of changing the transmembrane ratio of their substrate concentrations (properly, activities) away from 1. Those lacking secondary coupling are referred to as uniporters, symporters cotransport co-substrates, while antiporters act to exchange substrates in opposite directions in a coupled manner. "Group transfer" reactions involve the direct coupling of a chemical motif to the substrate, as in the PEP-dependent glucose transferases, whose external substrate is glucose but whose internal product is glucose-6-phosphate.

It is notable the use and misuse of the term "passive" to describe transport activities; this term has two common but orthogonal meanings. The first is thermodynamic, and means "equilibrative,"

while the second is mechanistic and is then taken to mean “transport through a bilayer.” When the mechanism involves a transporter, it is properly known as “facilitated diffusion.” Since these two uses of “passive” are often conflated, and consequently cause much unnecessary confusion, we recommend that the term ‘passive’ is simply dropped in the context of transporters.

In a similar vein, it is common to refer to “influx” and “efflux” transporters on the basis of the direction of substrate flux observed in their most typical operating conditions. Clearly, however, any reaction is in principle thermodynamically reversible (even if free energy changes are large and negative). We note in particular therefore that while a particular transporter might “normally” be an “influx” transporter if its substrate is provided externally, there is no reason of principle, especially if it is equilibrative, why it would not become an “efflux” transporter if large amounts of the same substrate are made intracellularly in a biotechnological process. Consequently, while the focus is on ‘efflux’ transporters, we shall have plenty to say about the more widely studied “influx” transporters as well.

The first step in a systems biology strategy is to make a model of the organism of interest, and discover which transporters might have the desired activity, and whether native activities can be increased or if it is necessary to add exogenous genes.

There can be sound evolutionary (natural selection) reasons why a cell might naturally choose to efflux expensively synthesized product; the biotechnologist is wise to make use of these where they exist. By contrast, for substances that are actively biosynthesized by the host, it is rather less obvious why they might evolve an efflux transporter for them instead of simply lowering the rate of synthesis to a level that is adequate to satisfy the requirements of the host. Needless to say, evolution has in fact selected for this active efflux, and it is of interest to seek to understand its basis (if only to replicate it in the selection schemes of the biotechnologist).

In favorable cases, it may be possible to pump out the product of interest using an efflux transporter that is coupled to cellular sources of free energy. The methods of synthetic biology offer almost unlimited opportunities for efflux transporter engineering, and thereby for learning the sequence-structure-activity relationships of transporters and their substrates of interest.

Biorefineries

Fossil fuels have been industrialising the world for hundreds of years. According to International Energy Outlook 2016 (EIA, 2016), total world consumption of marketed energy expands from 549 quadrillion British thermal units (Btu) in 2012 to 629 quadrillion Btu in 2020 and projected to 815 quadrillion Btu in 2040. This shows a 48% increase from 2012 to 2040 (EIA, 2016). With the increasing fossil fuels price and depleting natural resources, many researchers are searching for sustainable alternative energy sources. The "450 Scenario" envisioned by the IEA is a pathway to limit long-term global warming to 2°C above pre-industrial levels, i.e., place stringent limits on how high atmospheric CO_2 concentrations are allowed to rise (< 450 ppm). To achieve this, a range of specific policies is invoked, including carbon price, the removal of subsidies for fossil fuels and their derivatives, greatly improved efficiencies of medium- and heavy-duty vehicles, internationally approved and imposed CO_2 emission limits for new cars and aircraft and support for renewable technologies (International Energy Agency, 2016).

This should (logically) have acted as a driver for biofuels to substitute conventional fossil fuel use but three counteracting factors have become important:

1. The perceived conflict between food production and land use for growing crops suitable for biofuels production.
2. The environmentally damaging effects of massive first-generation biofuels production.
3. The rapid move to national policies aiming at completely replacing liquid fuel-dependent vehicles by electric vehicles.

The large-scale production of the so-called first-generation biofuels (ethanol and methyl esters of long-chain fatty acids) is a mature and global industrial sector using mostly food crops (corn and sugarcane) for ethanol and a widening portfolio of plant and animal fats and oils for the methyl esters that constitute biodiesel. In addition,

a series of other “advanced biofuels” molecules have established production routes: n-butanol, isobutanol, 4-methyl-pentanol, n-pentanol, and so on. All these chemical entities can be intermediates in the biosynthesis of higher-value bioproducts; in other words, decades of biotechnological and bioengineering experience with biofuels are not to be discarded but should be seen as a platform for further technological R&D in biorefineries for a wide spectrum of industrial, agricultural and consumer chemicals.

Biorefineries are expected to effectively utilize abundant biomass resources in a sustainable manner in order to ensure energy security, mitigate climate change, and meet the endless demand for chemicals and materials. A schematic description of the biorefinery processes is shown in Figure 8.

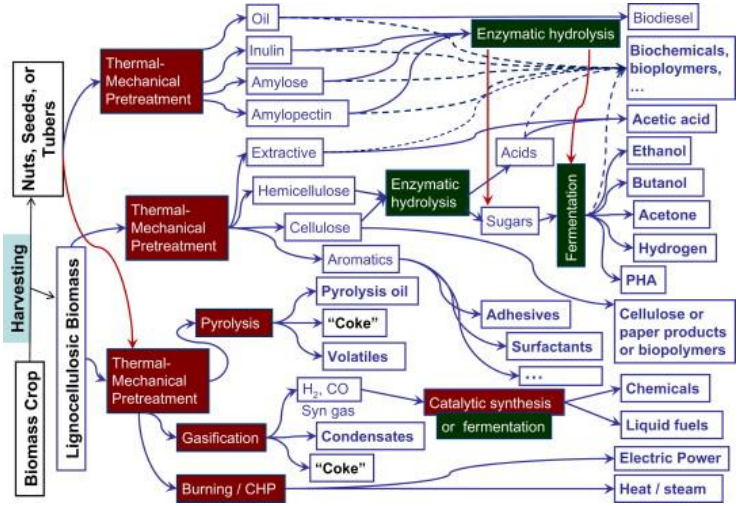

Figure 8. A schematic description of biorefinery processes.

Various types of biorefinery have been presented in the literature. Most of them are mainly defined based on the individual feedstock, such as corn-based biorefinery, wood-based biorefinery, forest-based biorefinery, palm-based biorefinery, algae-based biorefinery, etc. On the other hand, some researchers and technologists defined biorefinery

based on the generation of feedstock, which are first generation biorefinery (energy crop, edible oil seeds, food crops, animal fats, etc.), second generation biorefinery (lignocellulosic biomass) and third or fourth generation biorefinery (algae and other microbes). However, to further enhance the efficiency of such biorefinery, the concept of integrated biorefinery which focuses on the integration of various biomass conversion technologies is proposed. Within integrated biorefinery, multiple feedstock can be used to generate various types of products.

Biorefineries can be generalised into two categories: Energy-driven and Product-driven. In the Energy-driven Biorefinery, the main target is production of biofuel and bioenergy. Meanwhile, Product-driven Biorefinery is focusing on production of bioproducts (food/feed/chemicals/materials). Meanwhile, the bioenergy will also be produced simultaneously during the production of bioproducts. Figure 9 shows the classification of biorefinery based on "biomass to end product chain" (IEA Bioenergy, 2009).

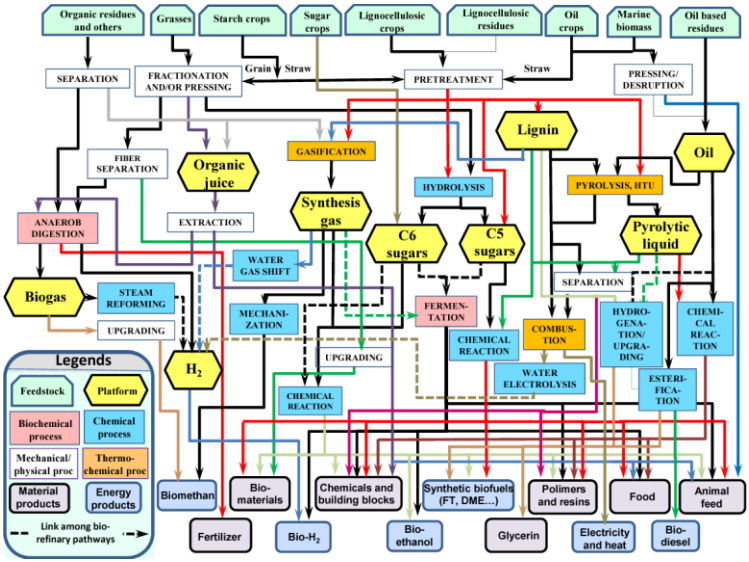

Figure 9. *Classification of biorefinery system (IEA Bioenergy, Task 42).*

The major economic challenges for biorefineries beyond the demonstration and pilot scales have resulted in three radically different models for biorefineries. The first is driven by the aim to decarbonize the global economy for environmental reasons and addresses the question of the complete replacement of petrofuels and petrochemical feedstock from renewable plant biomass sources. The second looks to tap the vast resources of marine resources to overcome the logistical hurdles of transporting large but unpredictable quantities of biomass from primary producing zones to centralized terrestrial biorefinery sites. The third seeks to directly utilize CO_2 in the atmosphere or from industrial processes as the carbon substrate.

Bioreactors

The bioreactor is one of the important components of industrial microbiology where microorganisms are being cultivated in such a way to their optimal physico-chemical and nutritional levels for the production of variable microbial products via specific fermentation process (Fig. 10). Variety of microorganisms of different nutritional classes having different mode of metabolic process along with the requirement of air, pH and temperature change during fermentation are regulated through bioreactor systems depending on the nature of microorganisms. The initial steps in the development of bioreactors status with the knowledge of microbial growth kinetics along with nutritional categories and the rate of their uptake are essential. The nutrient from the environment must be transported across the cell membrane into the cell. This is often the rate limiting step in the conversion of raw materials to the products and therefore, is important in assessment of a fermentation process.

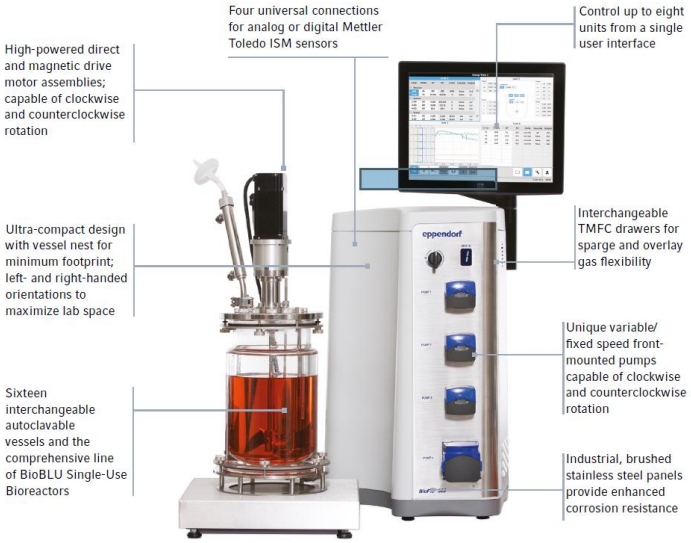

Figure 10. Structure of Bioreactor

Bioflo 320 structure in 360^{O}

Bioreactor systems categorization at basic level is mainly based on the process approach **submerged** as well as **solid state/solid** substrate fermentation. Both the approach dependent upon the nature of microorganism mainly at their water activity (a_w).

The main subdivisions of a standard stirred-tank reactor (STR) are

- A **base unit** connected to pipe work components for control of temperature, stirring, gassing, and additions of reagents
- A **culture vessel** with fittings and ports to aid gas transfer, liquid addition/removal, mixing, sampling, and fitting of sensors
- **Peripheral equipment** such as reagent containers, additional sensors, and special systems for sterilization, cooling, separation, and removal of culture constituents
- Instrumentation for measurement and/or **control of key process parameters** with links to supervisory software and remote control facility

Measurement and Control System of Bioreactors

Schematic overviews and descriptions for key parameters control loops as used primarily in bench scale bioreactors are provided below (Fig. 11, 12).

Speed control relies on the feedback from a tachometer located within the drive motor. Actual speed in revolutions per minute is displayed, as determined by the tachometer signal. A power meter is sometimes included and indicates how hard the motor has to work to maintain the set speed and, thereby, indirectly, the viscosity or "density" of the culture fluid. Speed range is typically from 50 to 1,500 rpm for bacterial systems and 10–300 rpm for cell culture units. It is now possible to use "universal motors" that can cover the complete range of speeds for microbial and cell culture applications for bench-sale bioreactors. Where speed is used to control the level of dissolved oxygen, an external signal from the oxygen controller can influence the stirrer speed. In this case, an absolute maximum and minimum value for speed can be set on the speed control module to limit the effects of the oxygen controller.

A thermos-circulation system around a vessel jacket has been chosen as an example here because it is the most complex of all of the methods of **temperature control**. For direct heating such as via a heater pad, it is simply a matter of fitting the heater, setting the desired temperature, and switching on. Cooling is normally via a cold finger and flow of cooling water is controlled via the action of a solenoid valve. The Pt-100 sensor provides the feedback signal, which causes the controller to take one of the following actions:

- Heat at full power because the actual temperature is some way below the set point.
- Pulse the heater power because the actual temperature is close to set point.
- Turn on the cooling valve because the actual temperature is above set point.

There is usually some indication to show which action the controller is taking at any given moment. A circulation pump and pipe work are added to the system for water circulation, and any heating is indirect (i.e., on the water circulating in the vessel jacket and not direct heating of the culture). In this case, a connection to a cold-water supply must be made (securely, using jubilee clips or cable ties).

Use of chillers for cooling water circulation is becoming more common and this usually requires the addition of a bypass and pressure relief valve to accommodate the cooling water valve being closed at the bioreactor. Unless the cooling system has a large cooling capacity, it will be unsuitable for rapid cooling after vessel sterilization, so a supply of house water may still be needed.

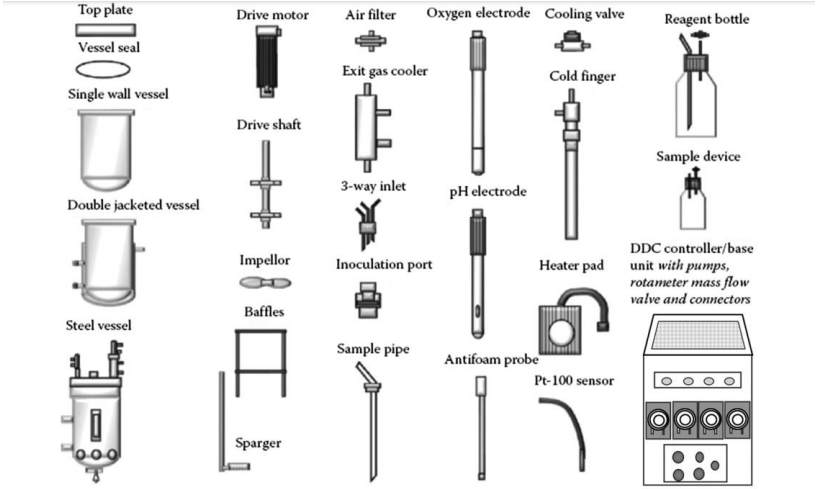

Figure 11. Major components of a bioreactor.

The heating and cooling is controlled in the same way as a directly heated system, but only the water in the jacket is affected. The jacket provides a large surface area in contact with the vessel wall for heat exchange. Good temperature control can be achieved from approximately 5–8°C above the ambient temperature or above the

temperature of the cooling water. Counter cooling with water ensures stable temperature control when operating near ambient temperatures. Measured range is typically from 0 to 60°C (exceptionally up to 90°C).

Gas control is essential for the bioreactor work. A compressed gas (normally oil-free air) is supplied to the bioreactor at a maximum of 0.5–0.75 bar. The rotameter controls the actual flow rate of air through the bioreactor. This should not exceed 1.5 vessel volumes per minute otherwise droplets of water may be carried out with the gas leaving the bioreactor, thus wetting the exit gas filter, which in turn causing it to block. A valve at the bottom of the rotameter is turned and the indicator ball in the rotameter tube rises or falls in proportion to the valve position. A scale on the tube gives flow rates in milliliters per minute or liters per hour. The air passes through the inlet air filter, which prevents any microbes from entering the vessel via this path. The use of several impellors ensures that all regions of the vessel receive good aeration. A "headspace" of approximately 20–30% is normally left between the culture level and the vessel top plate. Sometimes, gas can also be introduced into this region via a short pipe in the bioreactor top plate (e.g., CO_2).

The hydrogen ion concentration **(pH) is controlled** by the addition of acid or alkali as the conditions change with growth. The controller uses a pH electrode (typically a gel type) to sense these pH changes and provide a feedback signal, which activates the supply of acid or alkali to bring the pH back to the set point. The latest generation of probes can store calibration data, and so on, within the prober and can communicate via a serial protocol such as Modbus. The pH meter is calibrated before the electrode is autoclaved. Steamsterilizable electrodes have a limited life cycle (20–50 sterilizations cycles). The pumps supplying the acid and alkali are normally built into the instrumentation or base unit housing.

Figure 12. Sammary screen of operation system: 1. Loop name, 2. Mode, 3. Setpoint, 4. Process value, 5. Units, 6. Cascade, 7. Output

The reagent bottles are connected to the bioreactor via silicone tubing; the bore size of which determine the volume of acid or base that can be added when the pumps are turned on. Selecting the concentration of the acid or alkali will determine how much effect each dose has on the vessel contents. Normally, the concentration of acids and alkali are in the region of 0.5–2 M. The use of ammonium salt as an alkali has the added advantage of adding extra nitrogen for the growing culture.

Care should be taken when using ammonia water because the ammonia can become gaseous in the tubing. A set-point value as well as an upper and lower limit is fed into the controller to provide a “dead band” range in which the controller is inactive. This band is normally ±0.5 pH units of the desired value. A proportional band adjustment may be present to widen or tighten the range of pH value over which the controller acts.

Dissolved oxygen is one of the most difficult parameters to control. The electrodes used to measure dissolved oxygen are commonly of the polarographic type, which respond rapidly, robustly, and accurately to changes in the oxygen concentrations. The key point with this type of electrode is that it requires a voltage to polarize the anode and cathode of the detecting cell. This polarization can take between 2 and 6 h to complete. During this time, the electrode must be connected to its relevant module, which in turn must be switched on. The latest generation of dissolved oxygen probes use a fluorescence technique which does not require any polarization time. These probes can store data regarding calibration and communicate with the bioreactor controller via a serial protocol such as Modbus. To set the electrode to zero after autoclaving, first pass oxygen-free nitrogen through the culture vessel for a few minutes and once all oxygen is expelled, set the zero point. The 100% value is a relative setting made after autoclaving and polarization of the electrode by turning on the airflow and stirrer speed to the maximum speed needed for a few minutes and then adjusting the controller to display 100%. Both types of electrode have a consumable component, which needs to be replaced periodically and a special cartridge kit is available from the manufacturer to make this a simple task. Control of dissolved oxygen can simply be achieved through influencing speed control, adjusting airflow, or by a combination of both. Increasing the speed of mixing and/or airflow may increase foaming to a level that becomes problematic. The most accurate form of flow control is to use a thermal mass flow control valve, which measures and controls airflow based on the cooling effect the gas exerts when passed over a heated element.

A conductance-type probe that is fitted in the vessel headspace detects the formation of foam. Once foam is detected, the probe gives the **controller** the signal to dispense a dose of **antifoam**. A delay timer ensures the antifoam reagent has adequate time to reduce the foam level before another dose of antifoam is added. The sensitivity of

foam detection should be adjusted to suit the conditions prevailing in the bioreactor. A sheath of inert material around the probe prevents splashes of foam from giving "false positives". Normally, the metal top plate is used to provide the electrical circuit for the probe to operate so a flying lead is provided that fits into a socket somewhere on the top plate. Antifoam reagents can be mineral oils, vegetable oils, or certain alcohols. Commercial preparations are available for use in pharmaceutical fermentations. The key thing with using oils is that they can form a skin on the surface of the culture and interfere with gas transfer at the liquid/air interface. If foam builds up unchecked, then it can reach the exit gas filter, blocking it and providing a path for contamination.

It is essential the **reduction/oxidation (redox) potential** of a system or a chemical, usually expressed relative to a standard hydrogen half-cell with a redox potential of 0.00V. It follows that a reducing half-cell will have a negative value whereas an oxidizing agent will have a positive one. Oxidation/reduction reactions are generally reversible. Although the redox value obtained during aerobic fermentation is not generally informative because of the complex nature and multiplicity of biochemical reactions, the opposite is true in anaerobic fermentations because the redox electrode, and, in turn, the value obtained, is very sensitive to oxygen, thus providing a safety indicator for anaerobiosis; a redox value below –200 mV is a good indicator of anaerobic conditions. The redox electrode closely resembles a pH electrode and the conditions for handling and care are almost identical. An electrical zero can usually be set and shorting out the electrode connections should give a reading close to 0 mV. However, pH buffer 4 should never be used with redox electrodes because it will cause the electrode to malfunction and soaking for 24 h in electrolyte may be needed to restore the probe's function.

There are several additional parameters that can be measured and, in turn, controlled during the fermentation, such as airflow, weight, pressure, biomass, optical density, exit gas analysis etc.

Fermentation in laboratory. Bioprocess run

A bioprocess run typically comprises the following steps.

1. Preculture: The medium in the bioreactor is inoculated with a preculture. Often, the preculture is grown in a shaker or incubator. Sometimes smaller bioreactors are used to grow precultures for the inoculation of larger bioreactors.
2. Bioreactor preparation: The bioreactor is prepared in parallel to inoculum preparation. Preparations include the sterilization of bioreactor, feed lines, and sensors; medium addition to the bioreactor; the connection of the bioreactor with the bioprocess control station; and the definition of process parameter setpoints in the bioprocess control software.
3. Inoculation: Once the bioreactor is prepared, the medium is inoculated.
4. Cultivation period: During the cultivation period, agitation, pH, temperature, and DO are typically monitored and controlled in real time via the bioprocess control software. In addition, scientists often take culture samples to analyze, for example, the biomass and the concentration of metabolites. Eventually researchers feed the culture by adding nutrient solutions.

Eppendorf BioFlo®320 Webinar

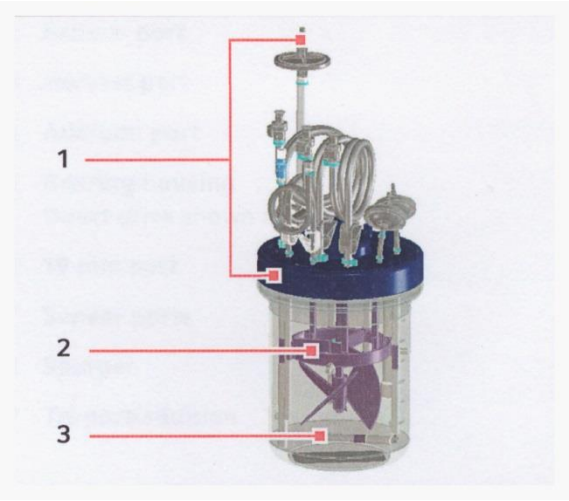

Figure 13. Simple structure of Bioreactor: 1. Headplate and tubing assemblies, 2. Vessel, 3. Internal components

5. Culture harvest: Scientists typically end the bioprocess run and harvest the culture when it enters the stationary growth phase.
6. Downstream processing: The culture broth is further processed.
7. Bioreactor cleaning: The bioreactor is sterilized to inactivate culture residues and cleaned.

Preparation of Bioreactor to Use

1. **Disassembly of the Vessel**. The fermentation is shut down from the control unit and transfer lines plus cable connections removed (See Fig. 13). Reagents lines are emptied and may be refilled with water. After fermentation, the vessel and reagent tubing should be re-autoclaved, ensuring that inlets and outlets are properly prepared.
2. **Cleaning**. The pH and dissolved oxygen electrodes should be removed and stored in suitable reagents, as described in the manufacturer's instructions. Periodic cleaning and regeneration of the electrodes are also covered by these instructions. Vessels must be stored clean and dry.
3. **Preparations for Autoclaving.** The vessel seal should be removed checked for damage and can be dipped in water to aid relocation. The minimum medium volume is the amount needed to adequately cover the electrodes. The vessel top plate can now be replaced and any clamping ring or bolts tightened firmly. The ports for electrodes have O-ring seals, which should be checked for damage and may be wetted with a little water to aid relocation (Fig. 14). The pH electrode should be calibrated in appropriate buffers for the usual two-point calibration. The pH and dissolved oxygen electrodes should be fitted, taking care not to damage them by careless insertion into the port. For the dissolved oxygen electrode, a cap may have to be improvised from aluminum foil. The Pt-100 temperature sensor must be fitted and capped unless it fits into a pocket and so can be removed totally. If used, the foam probe is fitted so that it is above the liquid level. A foam probe can be pulled out of a vessel after autoclaving with little risk of contamination but cannot be pushed down. If the vessel has a conventional rotating mechanical seal, the lubricant

reservoir must be checked and topped up with a suitable reagent if necessary (usually glycerin).

Reagent bottles are prepared in a similar way to the bioreactor vessel. A cap or head plate (including a seal) is fitted with a short tube and longer dip tube. A disposable filter is then connected to the short tube with silicone tubing. The shorter pipe must not dip into the liquid, and nothing must block the free passage of air through the filter. The long pipe dips into the liquid as far as possible, usually with a plastic/silicone tubing extension. This pipe should be fitted with a length of silicone tubing that is long enough to reach the peristaltic pump. The tubing is clamped so that no liquid can escape during autoclaving. The exit gas cooler should be fitted to one of the larger available ports. A short length of silicone tubing should be attached to the top of the air outlet, and a small 0.22- or 0.45- μm filter fitted.

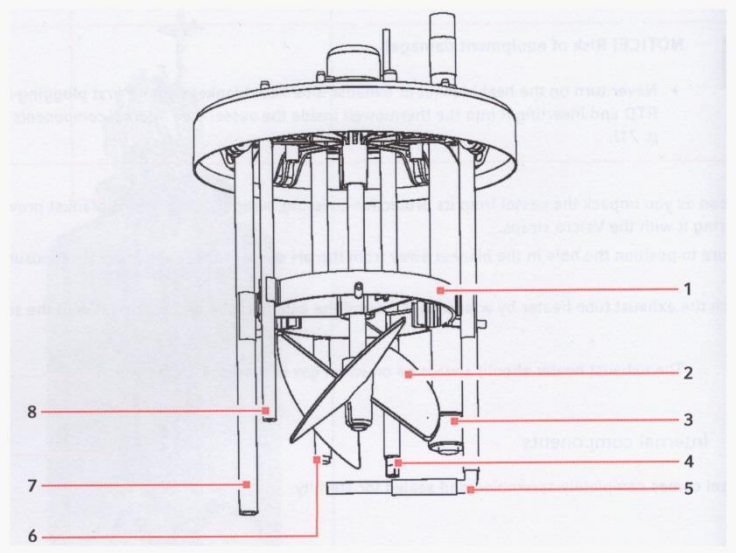

Figure 14. Internal vessel Components: 1. Support ring, 2. Pitched blade impeller, 3. DO tube with silicone cap, 4. pH tube, 5. Porus microsparge or tube macrosparge depending on configuration selected, 6. RTD thermowell, 7. Harvest tube, 8. Sample tube.

The air outlet line must be kept open during autoclaving. A short length of silicone tubing must be fitted to the air sparger inlet pipe with a 0.2-μm disposable filter mounted on top. The tubing between the sparger pipe and the filter must be clamped shut during autoclaving. If a port is to be used for inoculation or piercing with a needle, a silicone membrane must be fitted into the empty port and a clamping collar/cap used to hold it in place.

4. **Autoclaving.** The vessel and any reagent/sampling bottles already connected by silicone tubing are assembled together on a steel tray or in an autoclave basket. A final check should be made that at least one route is available for air to enter and leave the vessel(s) and that all lines dipping into liquid are clamped closed. If the vessel has top drive and a mechanical seal, the seal must be lubricated (normally with glycerin). If the medium cannot be autoclaved, a suitable volume of distilled water should be used (e.g., 10–20 mL/L of working volume to keep the electrodes wet). A quantity of liquid is certain to be lost during autoclaving (~10%) so the medium is over-diluted to compensate for this or sterile distilled water is added afterward to restore the volume. Some form of indicator such as autoclave tape should be included to provide a warning if the correct sterilization procedure has not been carried out. Autoclaving at 121°C for a minimum of 30 min up to 1 h is normally considered adequate for vessel sterilization but consider potential damage to the constituent chemicals of the medium.
5. **Setup after autoclaving.** The air sparger is connected to the rotameter by a piece of silicone tubing from the top of the filter to the air outlet of the rotameter (Fig. 15). The air sparger line is unclipped between the metal pipe and the air filter. The exit gas cooler is connected to the water supply directly or via the bioreactor base unit. The tubing for water in, water out, and drain is connected to the vessel jacket for a

water system, and the water is turned on so that the vessel jacket is filled. Alternatively, any pads or heater cartridges are connected to the base unit or temperature control module; the cold finger is connected to the water supply. The tubing from the reagent bottles is connected to the multiway inlet (if necessary), and the silicone tubing from the reagent bottles is located in the relevant peristaltic pump. Any aseptic connections must be made first if the reagent bottles were autoclaved separately from the vessel. The clamps are removed so liquid can flow freely. A manual switch is often fitted, which allows the pumps to be primed with liquid before use. The drive motor is located onto the top plate (if appropriate), ensuring a good connection is made to the drive shaft. The Pt-100 temperature sensor is connected to the control module and removing the shorting cap and screwing in the cable connect the pH electrode. The dissolved oxygen electrode is connected to the appropriate cable (this requires some care, but the connector should lock firmly when it is correctly positioned by aligning the marks on the connector collar and the probe top).

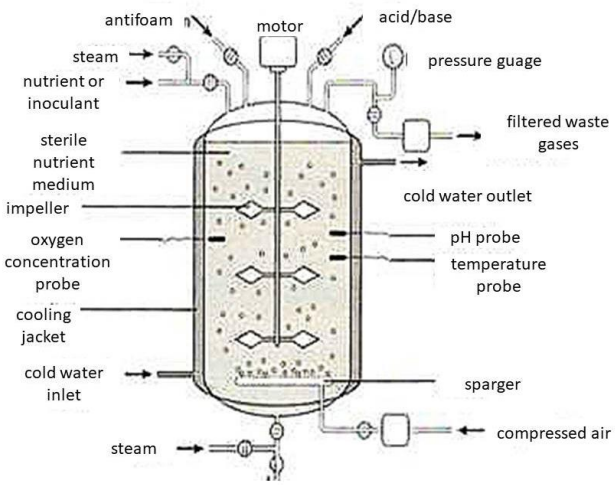

Figure 15. Batch Bioreactor Design

Connections to the foam probe are made, usually one wire into the electrode and one on the vessel top plate to make a circuit. If necessary, the dissolved oxygen electrode should be polarized. Setting the temperature control at this stage will ensure the bioreactor is ready to inoculate after calibration of the dissolved oxygen electrode. The dissolved oxygen electrode is calibrated for the zero point using nitrogen gas and then the air supply is turned on. The maximum stirrer speed to be used and the maximum airflow required on the rotameter are set. After leaving for approximately 15 min, the 100% level is set. The bioreactor is now ready to inoculate.

6. **Inoculation of the Bioreactor Vessel.** The air sparger is connected to the rotameter by a piece of silicone tubing from the top of the filter to the air outlet of the rotameter. The air sparger line is unclipped between the metal pipe and the air filter. The exit gas cooler is connected to the water supply directly or via the bioreactor base unit. The tubing for water in, water out, and drain is connected to the vessel jacket for a water system, and the water is turned on so that the vessel

jacket is filled. Alternatively, any pads or heater cartridges are connected to the base unit or temperature control module; the cold finger is connected to the water supply. The tubing from the reagent bottles is connected to the multiway inlet (if necessary), and the silicone tubing from the reagent bottles is located in the relevant peristaltic pump. Any aseptic connections must be made first if the reagent bottles were autoclaved separately from the vessel. The clamps are removed so liquid can flow freely. A manual switch is often fitted, which allows the pumps to be primed with liquid before use. The drive motor is located onto the top plate (if appropriate), ensuring a good connection is made to the drive shaft. The Pt-100 temperature sensor is connected to the control module and removing the shorting cap and screwing in the cable connect the pH electrode. The dissolved oxygen electrode is connected to the appropriate cable (this requires some care, but the connector should lock firmly when it is correctly positioned by aligning the marks on the connector collar and the probe top). Connections to the foam probe are made, usually one wire into the electrode and one on the vessel top plate to make a circuit. If necessary, the dissolved oxygen electrode should be polarized. Setting the temperature control at this stage will ensure the bioreactor is ready to inoculate after calibration of the dissolved oxygen electrode. The dissolved oxygen electrode is calibrated for the zero point using nitrogen gas and then the air supply is turned on. The maximum stirrer speed to be used and the maximum airflow required on the rotameter are set. After leaving for approximately 15 min, the 100% level is set. The bioreactor is now ready to inoculate.

7. **Sampling from a Bioreactor Vessel.** All sampling starts with a sample pipe, which should dip into the bulk of the culture liquid. At its simplest, a sampling device consisting of a bottle

connected to two metal needles/pipes permanently fixed through the metal and rubber seals of its cap. One pipe is connected to a 0.22-μm-air filter, whereas the other, which is linked by silicone tubing (clamped off until a sample is needed), is connected to the sampling pipe. A syringe is fitted to the air filter after autoclaving. The sample device is usually attached to the vessel top plate so that the glass bottle hangs down vertically beneath the cap and a supply of bottles of the same size are autoclaved ready for use. The use of disposable sterile syringes and one-way valves manufactured for medical applications can now make an inexpensive, reusable, and reliable sampling system for bench scale bioreactors. Resterilizable sampling systems for ISS vessels provide similar functions in large-scale industrial bioreactors.

Laboratory experiment 1
Batch fermentation. Control set-points for *E. coli*.

E. coli is a gram-negative, non-spore-forming, facultative anaerobe bacteria that has a long history in the world of biotechnology and laboratory work due to its ease of manipulation and well-understood genetic system. Fast doubling time of 15 - 20 minutes makes it an excellent model organism for research and production.

The method describes the determination of suitable parameters and set-points for the operation of the bioreactor Eppendorf BioFlo 320 during fermentative growth of *Escherichia coli*. This method includes preparation of the growth medium, regulation and control of medium condition for molecular hydrogen production.

Instruments and equipment

Bioreactor Eppendorf BioFlo 320, Thermostat, Autoclave, pH-meter, Pipettes, sterile filter 0.22 μm.

Chemicals, solutions or necessary supplies

Peptone, K_2HPO_4, KH_2PO_4, NaCl, glucose, NaOH, pH standard buffers pH 4.0, 7.0, 10.0.

Description of the measurement procedure

Prepare Bioreactor for the experiment as described in the section Preparation of Bioreactor to Use**.**

E. coli growth medium is prepared and poured into the vessel for a fermentation run. Vessel volume should be reserved for components, including inoculum, and other temperature-sensitive material which will be added after sterilization.

1. Prepare 4330 ml high buffered peptone medium for bacteria growth according to the Table 1

Table 1. Medium composition

Chemicals	**Concentration (g L^{-1})**
peptone	**20**
K_2HPO_4	**15**
KH_2PO_4	**1.08**
NaCl	**5**

Medium pH was determined by pH-meter via selective pH-electrode (HI1131, Hanna Instruments, Portugal) and adjusted by 0.1 M NaOH or HCl.

2. Prepare 9 % 100 ml glucose sterile solution. After autoclaving and allowing time for the vessel to cool addition of temperature-sensitive material can be made:

Chemicals	**Concentration**
glucose	**100 ml 9 %**

3. Prepare 10 % NaOH for pH control.
4. Prepare inoculum 70 ml using high-buffered peptone medium composition. The bacteria stored in a glycerol solution (75% glycerol, 24% 0.1 M NaCl ы 1% 1 M MgSO4) in a 80 ^{0}C freezer (Biobase, China) were transferred on a Petri dish containing a solid nutrient medium, which was stored at 3-5 ^{0}C and used during experiments. The bacteria transferred to a liquid nutrient medium were cultivated at 37^{o}C for 20 h.

After addition of inoculum take samples every hour.

The set-points for *E. coli* are entered into the bioprocess control software prior to inoculation. Except for dissolved oxygen (DO) which remains zero, the medium should be allowed to equilibrate prior to inoculation. An initial DO value of 0 % is acceptable. Set-points are commonly controlled in either automatic mode or via a cascade (Table 2).

Table 2. Set-points for bioprocess control.

Parameter	**Setpoint**
Temperature	37 ^{0}C
pH	7.5
DO	0 %
Agitation	300-1200 rpm

pH control

pH control often uses the addition of liquid acid and liquid base solution to maintain pH at setpoint, but often it relies on the acid/base-producing properties of the culture or medium for a natural drift up or down. *E. coli* pH control is usually done through the addition of base (10 % NaOH). Typically systems will allow the user to assign pumps a specific function such as acid or base. When a deviation in pH calls for an adjustment, the specified pump will turn on until the deviation no longer exists.

pH calibration

pH calibration is usually done outside the vessel using a two-point calibration method and standard buffers. Buffer 7.0 is commonly used for the Zero and either 4.0 or 10.0 is commonly used for the Span. pH is calibrated prior to autoclaving.

Dissolved oxygen (DO) sensor calibration and gassing control

Use an analog polarographic DO sensor calibration via a standard two-point calibration method: 0 % (set “ZERO”) is obtained by disconnecting the sensor from the cabinet and allowing the raw value to stabilize; 100 % (set “SPAN”) is obtained by running different agitation speeds for BioFlo 320 5 L run 711 rpm, and 1.5 VVM 7.45 SLPM air flow until the DO value stabilized at the maximum.

ORP calibration

Before using, the potential of the electrodes is measured in a test solution containing 0.049M $K_3[Fe(CN)_6]$ and 0.05M $K_4[Fe(CN)_6]\cdot 3H_2O$ (pH 6.86). The redox potential in this solution was 254 ± 10 mV at 25 ^{0}C.

Laboratory experiment 2
Continuous fermentation and feeding protocol

Instruments and equipment

Bioreactor Eppendorf BioFlo 320, Thermostat, Autoclave, pH-meter, Pipettes, sterile filter 0.22 μm.

Chemicals, solutions or necessary supplies

Peptone, K_2HPO_4, KH_2PO_4, NaCl, glucose, NaOH, pH standard buffers pH 4.0, 7.0, 10.0.

Description of the measurement procedure

1. Prepare Bioreactor for the experiment as described in the section Preparation of Bioreactor to Use**.**
2. Prepare growth medium as described in the Laboratory experiment 1
3. Prepare 1 L concentrated feeding medium composed from peptone 20 g L^{-1}, glucose 10 g L^{-1}. After preparation sterilize solution using sterile filter.
4. Prepare 10 % NaOH for pH control.
5. Prepare inoculum medium as described in the Laboratory experiment 1

Use 90 % of the vessel maximum working volume. *E. coli* is cultured in a chemically defined medium of pH 7.5. Add the initial fermentation medium to the vessel for sterilization. After the medium was cooled to growth temperature or room temperature, add sterile components. To maintain a constant working volume throughout the fermentation process, a continuous fermentation method is used, and volumes of *E. coli* culture identical to the volume of feeding medium added is removed upon feeding.

The feeding-in and pumping-out protocol shown in Table 3 illustrates the adjustments made to the pump speed over the course of the fermentation.

Table 3: Proportional feeding and broth removal strategy for continuous fermentation

Time (h)	Pump speed (mL/min)		Time (h)	Pump speed (mL/min)
3.5	0.35		8	4.4
4.5	0.65		8.5	5.15
5.5	1.00		10	17
6.5	1.95		24	20
7	2.65		48	25
7.5	3.35		72	30

Inoculum volume of 10 % of the initial fermentation medium volume is inoculated in the growth medium. Antifoam 204 (Sigma-Aldrich®, USA) is added only when foaming is observed.

pH calibration and control

pH sensors are calibrated outside the vessels prior to autoclaving them, using a two-point calibration method and standard buffers. Use the buffer of pH 7.0 to set "ZERO" and the buffer of pH 4.0 for the "SPAN"/"slope" (please refer to BioFlo user manuals). The pH is automatically maintained at 7.5 by adding 25 % (v/v) NaOH via a pump (assigned as "base"). The deadband for pH control set to 0.05.

DO sensor and ORP calibration are done as described in Laboratory experiment 1.

Laboratory experiment 3
Determination of bacterial growth properties

Instruments and equipment

Spectrophotometer, cuvettes.

Description of the measurement procedure

Measure optical density (OD) of the samples collected every hour via specrophotometer at 600 nm. Select the exponential growth phase and calculate the specific growth rate (μ, h^{-1}) according to the formula:

$$\mu = \frac{ln_{N2} - ln_{N1}}{t_2 - t_1}$$

where, the values ln_{N2} և ln_{N1}, respectively, were recorded in periods t_2 and t_1.

Example:

During the experiment results are the following:

Time	0	0.5	1	1.5	2	2.5	3	3.5	4	4.5	5
OD	0.0286	0.0386	0.0576	0.1621	0.3380	0.5211	0.6984	0.8690	1.0120	1.0680	1.0846

By placing the time-slip values on the graph, the growth curve of the bacteria is obtained, including the stationary phase. Taking the minimum and maximum values of the exponential phase in the 1st and 4th hours, respectively, placing them in the above-mentioned formula, we will get μ:

$$\mu = \frac{\ln(1.012) - \ln(0.0576)}{4 - 1}$$

$$\mu = 0.95\ h^{-1}$$

Mean generation time (T) can be also measured

$$T = 0.693/\mu$$

$$T=0.693/0.95$$

$$T=0.729\ h$$

Inverse value of the Mean generation time is growth rate (K)

$$K=1/T$$

$$K=1/0.729$$

$$K= 1.37\ h^{-1}$$

Laboratory experiment 4
Determination of utilization of substrates and generation of fermentation end-products

Instruments and equipment

High Performance Liquid Chromatography (HPLC), C18 column, RI and DA detectors, centrifuge, sterile filter (0.22 μm), Eppendorf type tubes, vials.

Chemicals, solutions or necessary supplies

HPLC grade glucose, formate, succinate, acetate, lactate, ethanol, H_2SO_4, acetonitrile, dd H_2O.

Description of the measurement procedure

Separation of compounds is performed on an Agilent 1260 Infinity II LC Bio-inert system using Macherey-Nagel EC 250/4.6 NUCLEOSIL 120-5 C18 column (250 x 4.6 mm, MN720041.46, Düren, Germany). The column is cleaned with acetonitrile/ water (80/20) at 60^{o}C overnight and then regenerated with the experimental conditions using 5 mM sulfuric acid as a mobile phase in ddH_2O for a few hours, which is ideally performed after each batch of analysis. For regular column maintenance, 20 μm inlet filter (5041-2168, Agilent, Germany) is used.

Sugars and alcohols are monitored using a refractive index detector (Agilent RID, G1362A) set on positive polarity and optical unit temperature of 55 ^{o}C with mobile phase in the reference cell, while organic acids are monitored using RID and/or ultraviolet detector at 210 nm (Agilent DAD WR, G7115A). A sample volume of 10 μL is injected into the column using a multisampler (Agilent Bio, G5668A) and the column temperature is maintained at 60 ^{o}C using a thermostatically controlled column compartment (Agilent MCT, G7116A).

Analyts are eluted isocratically with 5 mM H_2SO_4 at 0.4 mL/min for 42 min. Chromatograms are integrated using Agilent OpenLAB

CDS. Concentrations of dissolved substances are calculated according to standard outcomes.

Samples collected during bacterial cells growth are centrifuged with 3000 g (Thermo scientific Sorvall LYNX 6000, Germany). The supernatant is pre-filtered using 0.22 μm PVDF syringe filter to exclude bacterial cell existence in the analytes. 5 mM H_2SO_4 was added into each sample.

Example:

During the experiment results are the following:

Time	Glucose (mM)	Acetate (mM)	Lactate (mM)	Formate (mM)	Succinate (mM)	Ethanol (mM)
0	10.882	0.460	0.260	0	0.210	0.320
1	7.599	2.665	1.319	0.900	1.729	3.913
2	7.527	3.101	1.413	0.705	1.928	4.352
3	6.808	3.753	1.425	1.112	2.174	4.656
4	4.310	5.705	1.274	3.621	2.343	6.015
5	2.100	8.801	1.531	7.623	2.909	9.775
6	0.076	8.597	1.407	9.239	3.046	10.594
7	0.064	9.332	1.386	10.197	3.105	11.282
8	0.054	10.026	1.446	9.546	3.147	12.306
24	0.040	8.514	0.854	7.640	2.919	11.120
48	0	8.745	0.450	6.792	2.860	11.478
72	0	9.105	0.151	6.531	3.118	10.395

The data obtained through the HPLC are used to describe and regulate of metabolic processes. **Carbon balance** is one of the main characteristics of fermentation. Two different hours are selected to determine the carbon balance (A and B, where A < B). The amount of carbon (C, mM) is calculated. Carbon balance is expressed in %, where 100% is the total amount of carbon in A hour. Determination of

carbon balance is the basis for the study of bacterial physiology, a precondition for achieving high productivity from a practical point of view.

Calculation:

Calculate carbon balance during fermentation at 24 h.

A = 0 h

B = 24 h

Total C at A h = 10.882 mM x 6 (glucose) + 0.460 mM x 2 (acetate) + 0.260 mM x 3 (lactate) + 0 mM x 1 (formate) + 0.210 mM x 4 (succinate) + 0.320 mM x 2 (ethanol) = **68.472 mM**

Total C at B h = 0.04 mM x 6 (glucose) + 8.514 mM x 2 (acetate) + 0.854 mM x 3 (lactate) + 7.640 mM x 1 (formate) + 2.919 mM x 4 (succinate) + 11.120 mM x 2 (ethanol) = **61.386 mM**

$$\boldsymbol{Carbon\ balance} = \frac{61.386}{68.472}\ x\ 100\% = \mathbf{89.65}\%$$

Suggested CO_2 generated during fermentation is about 5 % of total carbon. As a result 5.35 % of substrate carbon is involved in the formation of biomass.

Carbon Conversion Efficiency (CCE) is one of the essential parameters and is determined by the following formula

$$\boldsymbol{CCE} = \frac{\Delta C_2}{\Delta C_1}\ \text{x}\ 100\%,$$

where ΔC_1 is the difference in carbon concentration (mM) of the substrate at a given growth period and ΔC_2 is the difference in carbon concentration (mM) of the fermentation product during the same growth period.

Calculation:

Calculate CCE of glucose to ethanol at 6 h.

ΔC_1 = (10.882 – 0.076) x 6 = 64.836

ΔC_2 = (10.594 – 0.320) x 2 = 20.548

$$CCE = \frac{20.548}{64.836}\ x\ 100\% = \mathbf{31.69}\%$$

Important characteristics of metabolism are the ***substrate utilization rate*** (R_{ut}) and ***generation of fermentation end-products*** (R_{pr}). These parameters are calculated by the following formula:

$$R_{ut} = \frac{C_1 - C_2}{t_2 - t_1}$$

$$R_{pr} = \frac{C_2 - C_1}{t_2 - t_1}$$

where C_1 is the concentration of the substrate or end-product (mmol) at the beginning of the process, C_2 is the concentration of the substrate or end-product (mmol) at the end of the process. t_1 is the beginning of the substrate utilization or end-product generation (h) and t_2 is the final time of the consumption of substrate or generation of end-product (h).

Calculation:

Calculate glucose utilization rate.

$$\boldsymbol{R_{ut}} = \frac{10.882 - 0.076}{6 - 0} = \frac{10.806}{6} = 1.801\ mmol/h$$

Calculate ethanol generation rate

$$\boldsymbol{R_{ut}} = \frac{12.306 - 0.320}{8 - 0} = \frac{11.986}{8} = 1.498\ mmol/h$$

Laboratory experiment 5
Determination of molecular hydrogen yield via Gas Chromatography

The gas chromatographic method for determining the molecular hydrogen yield allows direct measurement of the volume of molecular hydrogen released with high precision, unlike other methods for determining the hydrogen output.

Instruments and equipment

Compressor, N_2 and H_2 generators, GC syringe, Hangouts or Balch type tubes.

Description of the measurement procedure

Turn on the condenser, the nitrogen and hydrogen generators, then the GC and computer. To clean the tower, give the following instructions: injection front: 250^0C, front column: 2 ml/min flow rate, oven: 50^0C, TCD (front): 250 ^{0}C. Under these conditions, wait 20-30 minutes until the system is ready for operation.

Before injecting the sample, change the following settings: front inlet 150 ^{0}C, front column 1 ml/min, retention time 4 minutes, Post run 1 minute at 100 ^{0}C. In the case of sequential streams, select the *Sequence*, indicating the number and names of samples. Wait until the system is ready to work.

Fill the anode hermetically sealed vessel with hydrogen for calibration. Take 100 μl of hydrogen with a syringe, inject and start the flow. When setting the sequence, have the injectable sample ready: combine it with the next flow period that starts automatically. Carry out the same calibration for 200, 300, 500 μl of hydrogen. Match the integrals of the surfaces of the curves constructed with the resulting curves to the volume of hydrogen.

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