2.4 MEASUREMENT AND KINETICS OF MICROBIAL GROWTH

Microbial growth is described as an orderly increase in all chemical components in the presence of suitable medium and the culture environment. There are four types of microbial growth: bacteria grow by binary fission, yeast divide by budding, fungi divide by chain elongation and branching and viruses grow intracellularly in host cells.

2.5.1 MEASUREMENT OF MICROBIAL GROWTH

Growth of the cell mass or cell number can be described quantitatively as a doubling of the cell number per unit time for bacteria and yeasts or a doubling of biomass per unit time for filamentous organisms such as fungi. Measuring techniques involve direct counts, visually or using instruments and indirect cell counts.

The first method is to measure the dry weight of the cell material in a fixed volume of the culture by measuring the dry weight of the cell material in a given volume of the culture. The cells need to be removed from the medium and dried. Another method is to use the spectrophotometer to estimate absorbance of cell suspensions. The absorbance at a particular wavelength is proportional to the cell concentration. By plotting a standard curve of absorbance versus cell concentration, the cell concentration of an unknown sample can be calculated. Direct microscopic counts using a counting chamber can be used but this technique has limitations as the dead cells cannot be distinguished from living cells. Electronic counting chambers count numbers and measure size distribution of cells. These are more often used to count eucaryotic cells like blood cells. Indirect viable cell counts also called plate counts may be used. This involves plating out dilutions of a culture on nutrient agar. Each viable unit will form a colony and each colony that can be counted is called a colony forming unit (cfu) and the number of cfus is related to the viable count in the sample. Turbidity measurement is a fast and nondestructive method especially for counting large numbers of bacteria in clear liquid media and broths – but cannot detect cell densities less than 10^7 cells per ml. The biochemical activity may also be measured – e.g., O_2 uptake, CO_2 production, ATP production. This method requires a fixed standard to relate chemical activity to cell mass and/or volume.

Bacterial growth rates during the phase of exponential growth, under standard nutritional conditions (culture medium, temperature, pH etc.) define the bacterium's generation time. Generation times for bacteria vary from about 12 minutes to 24 hours. The generation time for *E. coli* in the laboratory is 15-20 min. Symbionts such as *Rhizobium* tend to have a longer generation time. Some pathogenic bacteria, e.g., *Mycobacterium tuberculosis* have especially long generation times and this is thought to be an advantage to their virulence.

When growing exponentially by binary fission, the increase in a bacterial population is by geometric progression. The generation time is the time interval required for cells (or population) to divide:

$$G = t/n$$

Where G is generation time, n is number of generations and t is time in min/hours

The equation for growth by binary fission is:

$$b = B \times 2^n$$

where b is number of bacteria at end of a time interval, B is number of bacteria at beginning of a time interval, n is the number of generations (number of times the population doubles in the time interval).

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

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

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

$$n = 3.3 \log b/B$$

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

2.5.2 GROWTH KINETICS IN BATCH CULTURE

Batch culture occurs in a closed system that contains an initial limited amount of substrate. The inoculated microorganism will pass through a number of growth phases (Fig. 2.2). During the log phase, cell numbers increase exponentially at a constant maximum rate. In mathematical terms, we can write:

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

where x is the concentration of microbial biomass, t is the time in hours and μ is the specific growth rate in hours^{-1}. If we integrate between time t_0 and time t_1 when the concentrations of the cells are X_0 and X_1 we obtain:

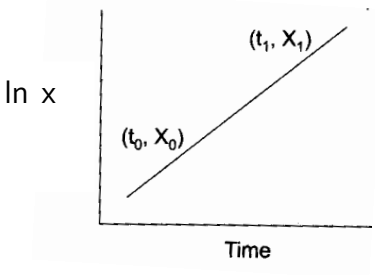
$$x_t = x_0 e^{\mu t}$$

where x_0 is the original biomass concentration, x_t is the biomass concentration after a time interval t hours and e is the base of the natural logarithm. On taking natural logarithms, the equation becomes:

$$\ln x_t = \ln x_0 + \mu t$$

Using this equation, a plot of the natural log of biomass concentration versus time should yield a straight line, the slope of which will equal the specific growth rate (μ).

$$\mu = \text{slope} = \frac{\ln (X_1 - X_0)}{t_1 - t_1}$$



During the exponential phase, nutrients are in excess and the microorganism is growing at maximum specific growth rate μ_{max} for the prevailing conditions.

The major problem of the exponential growth equation is that it does not predict an end to growth in a batch environment. According to this model, not only the whole earth, but also the whole solar system could become quickly covered by bacteria. However, growth results in the consumption of nutrients and the excretion of microbial products. After a time, the growth rate of the culture ceases. The cessation of growth may be due to the depletion of some essential nutrient in the medium (substrate limitation), the accumulation of some autotoxic product in the medium or a combination of the two.

The nature of the limitation of growth can be explored by growing the microorganisms in a range of substrate concentrations and plotting the biomass concentration in the stationary phase against the initial substrate concentration, the nature of growth limitation may be explored (Fig. 2.4).

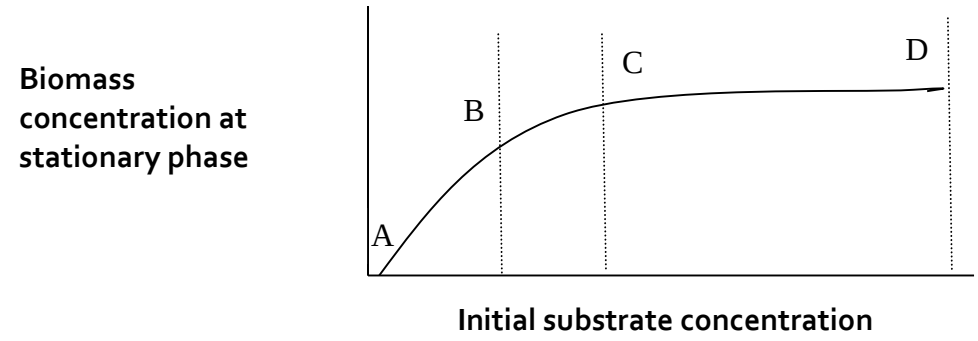


Fig.2.4: The effect of initial substrate concentration on the biomass concentration at the onset of stationary phase in batch culture (Stanbury et al. 1995).

A proportional increase in biomass is observed to increasing initial substrate concentration in the area between A and B which can be defined as:

$$x = Y (S_R - S)$$

Where x is the concentration of biomass produced, Y is the yield factor (g biomass produced per g substrate utilized), S_R is the initial substrate concentration and S is the residual substrate concentration. In the area between A and B, S is zero and therefore the equation above could be used to predict the biomass that could be formed from a certain amount of substrate. Between B and C although biomass increases with increasing substrate concentration, there is a diminished effect due to accumulation of toxic products or reduced availability of some other substrate. In the region between C and D, there is no change in biomass with increasing substrate concentration which may be attributed to increasing levels of toxic products or the exhaustion of some other substrate.

Y, the yield factor is the measure of efficiency of conversion of any one substrate to biomass. Although Y is not a constant and varies according to growth rate, pH, temperature, the limiting substrate and concentration of the substrate in excess, it can be used to predict the substrate concentration required to produce a certain biomass concentration. In the 1930s, Jacques Monod performed a number of initial rate experiments and plotted the specific growth rate against the concentration of growth-limiting substrate (Fig. 2.5). The result was a Langmuir type graph that appeared similar to enzymatic rate-substrate relationships described by Michaelis-Menton's model. Monod's model describing the relationship between the specific growth rate and the growth limiting substrate concentration is:

$$\mu = \frac{\mu_m S}{K_S + S}$$

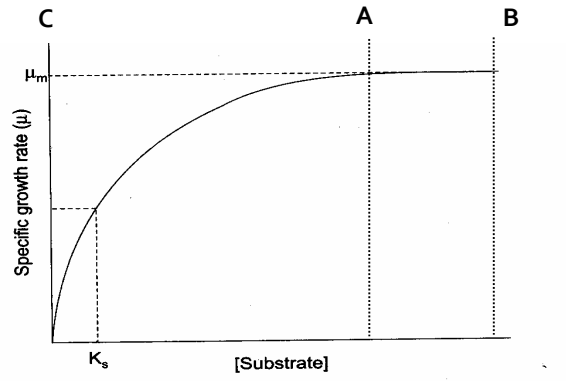


Fig.2.5: The effect of residual limiting substrate concentration on specific growth rate of a hypothetical bacterium (Stanbury et al. 1995, adapted).

Where μ_m is the maximum specific growth rate, S is the residual substrate concentration and Ks is the substrate utilization constant, numerically equal to substrate concentration when μ is half μ_m μ_m and is a measure of the affinity of the organism for its substrate. Zone A to B in Fig. 2.5 represents the exponential phase of growth in batch culture where substrate concentration is in excess and growth is at μ_m. Zone A to C is the deceleration phase, substrate concentration becomes limiting and cannot support growth at μ_m. An organism with a high affinity for the limiting substrate (low Ks) will have a short deceleration phase as the growth rate will only be affected when the substrate concentration is very low. Conversely, a microorganism with a low affinity for the substrate will have a very long deceleration phase (growth slows down at high substrate concentrations). The point when growth rate has declined to zero represents the stationary phase. This is a misnomer as many organisms are still metabolically active and are producing products called secondary metabolites during this phase.

Monod's model is widely used to describe the growth of many microorganisms. The equation adequately describes fermentation kinetics and can be used to describe complex fermentation systems. The equation adequately describes fermentation kinetics and can be used to describe complex fermentation systems, e.g., a commonly used expression to describe product inhibition is:

$$\mu = \left[1 - \frac{P}{P_m}\right]^n \frac{\mu_m S}{K_S + S}$$

Using the Monod model, a simple model microbial growth can be written as:

$$\frac{dX}{dt} = \mu X = \frac{\mu_m S}{K_S + S} X$$

$$\frac{dS}{dt} = -\frac{1}{Y_{xs}} \mu X = -\frac{1}{Y_{xs}} \frac{\mu_m S}{K_S + S} X$$

where Yxs is the biomass yield coefficient. The biomass yield coefficient is the efficiency of conversion of substrate to biomass and is calculated as:

$$\text{Biomass} = \frac{\text{Dry weight of biomass produced}}{\text{Weight of substrate used}}$$

The kinetics of product formation may be described as growth-linked products and non-growth linked products. In the first instance – these could relate to primary metabolites synthesized by growing cells and the non-growth-linked products would be secondary metabolites. Formation of growth-linked products can be defined by the following:

$$dp/dt = q_p x$$

where p is the concentration of product, q_p is the specific rate of product formation (mg product /g biomass/h).

Product formation can also be expressed in relation to biomass as:

$$dp/dx = Y_{p/x}$$

where $Y_{p/x}$ is the yield of product in terms of biomass (g product/g biomass). Combining these equations:

$$q_p = Y_{p/x} . \mu$$

Thus when product formation is linked to growth, the specific rate of product formation increases with specific growth rate and will be highest at μ_m. In this instance improved output will be obtained by increasing both biomass and μ. Non-growth linked product formation is related to biomass concentration. As these products are produced only under certain physiological conditions (usually limitation of a certain substrate), the biomass needs to be in the correct physiological state before secondary metabolites are produced.

Batch fermentations may be used to produce biomass and primary and secondary metabolites. For (i) biomass production: conditions supporting fastest growth rate and maximum cell concentration;
For (ii) primary metabolites: conditions to extend exponential phase accompanied by product excretion;
For (iii) secondary metabolites: conditions providing a short exponential phase and extended production phase or conditions giving decreased growth rate in the log phase resulting in earlier secondary metabolite production.

2.5.3 GROWTH KINETICS IN CONTINUOUS CULTURE

Exponential growth in batch culture may be prolonged by the addition of fresh medium, provided that the medium is designed to be substrate-limiting. If the vessel is designed with an overflow mechanism, such that the added medium displaced an equal volume of spent medium, then continuous culture of cells can be achieved. A steady state will be achieved if the medium is fed continuously at a suitable rate, i.e., formation of new biomass by the culture is balanced by the loss of biomass from the vessel. The flow of medium is related to the volume of the vessel by the **dilution rate** (D) as follows:

$$\mathbf{D = F/V}$$

Where F is the flow rate (l/h) and V is the volume (l).

The net change in cell concentration over time may be expressed as:

$$dx/dt = \text{growth} - \text{output} \quad \text{or} \quad dx/dt = \mu x - Dx$$

Under **steady state** conditions, the cell concentration remains constant, therefore

$$dx/dt = 0 \quad \text{and} \quad \mu x = dx \quad \text{and} \quad \mu = D$$

A continuous culture may be operated at dilution rates below the maximum specific growth rate and so within certain limits, the dilution rate may be used to control the growth rate of the culture. Cell growth in such a continuous culture is controlled by the availability of the growth limiting substrate and the system is referred to as a **chemostat**.

The mechanism underlying the controlling effect of the dilution rate is expressed in the Monod equation:

$$\mathbf{\mu = \mu_m s/(K_s + s)}$$

AT steady state, $\mu = D$

Therefore, $D = \mu_m \bar{s}/(K_s + \bar{s})$

where $\bar{s}$ is the steady state concentration of substrate in the chemostat.

Rearranging the equation:

$$\bar{s} = K_s D / (\mu_m - D)$$

which predicts that the substrate concentration is determined by the dilution rate. This occurs by the growth of the cells depleting the substrate to a concentration that supports the growth rate equal to the dilution rate. If the substrate becomes depleted below the level that supports the growth rate dictated by the dilution rate, the following would occur:

(i) The growth rate of the cells will be less than the dilution rate and they will be washed out of the vessel at a rate greater than they are being produced resulting in a decrease in biomass concentration.
(ii) The substrate concentration in the vessel will rise because fewer cells are left in the vessel to consume it.
(iii) The increased substrate concentration in the vessel will result in the cells growing at a rate greater than the dilution rate and biomass concentration will increase.
(iv) The steady state will be re-established.

Therefore a chemostat is a nutrient-limited, self-balancing culture system which may be maintained in a steady state over a wide range of sub-maximum specific rates.

The cell concentration in a chemostat is defined by:

$$\bar{x} = Y(S_R - \bar{s})$$

Where $\bar{x}$ is the steady state cell concentration.

By combining equation of steady state substrate and biomass concentrations:

$$\bar{x} = Y[S_R - \{K_s D/(\mu_m - D)\}]$$

Therefore biomass concentration at steady state is defined by operational variables S_R and D.
If S_R is increased, $\bar{x}$ increases but $\bar{s}$ remains the same.
If D is increased, μ will increase ($\mu = D$), $\bar{s}$ at the new steady state would have increased to support the elevated growth rate and less substrate will be available to be converted to biomass resulting in a lower $\bar{x}$.

An alternative type of continuous culture to a chemostat is a turbidostat. Here the concentration of the cells in the vessel is kept constant by controlling the flow of medium such that the turbidity of the culture is kept within certain narrow limits. To achieve this, biomass is monitored using a photoelectric cell, signals are sent to a pump controlling medium flow into the vessel. If the biomass exceeds a set point, the pump is switched on and if the biomass falls below the set point it is switched off. Other biomass measurement systems include CO_2 concentration or pH - biostat. However, the chemostat is the more commonly used system as it has the advantage over the biostat of not requiring complex control systems to maintain steady state.