3 Growth of Microorganisms

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1 Introduction

The capacity to grow, and ultimately to multiply, is one of the most fundamental characteristics of living cells. However, the definitions of growth are as broad as the scientific fields dealing with this topic.

Cell biologists are often interested in morphological changes of single cells especially during fission. Biochemistry investigates growth resulting from a few thousands of enzymatically catalyzed biosynthetic steps, organized in biochemical pathways, the kinetics of the growth process being therefore the overall kinetics of integrated enzymatic activity. From a biophysicist’s point of view, cells are open systems far from a thermodynamic equilibrium, exchange material and energy with their environment, and especially exhibit a large outflow of entropy. In chemical engineering, growth is referred to as an increasing amount of biocatalyst. Mathematical descriptions of growth are restricted to a couple of equations employing hyperbolic and exponential terms. Reduced to a common denominator, growth is usually considered as an increase of cell material expressed in terms of mass or cell number.

The objective of this chapter is, to give a general view of some aspects of growth of microorganisms. These aspects include modes of growth of single cells and cell aggregates, measurement of growth, limited to concurrent measurement of only some facets of biomass, dependence of growth on environmental factors and finally growth in bioreactors, influenced by man-made and somehow controllable impacts.

2 Modes of Growth

For the purpose of microbial growth quantitation, it is useful to consider some of the general growth properties of different classes of microorganisms and to attempt to establish some principles useful for interpreting measurements of their growth. The discussion of growth will focus on bacteria, yeasts, and mycelial molds. This grouping is chosen to reflect the mechanism of growth rather than usual phylogenetic lines. The rationale for this is that problems in quantitation of microbial growth are largely a result of the physical form of the microorganism, i.e., single cell versus mycelia, and the mechanism by which they divide, i.e., fission, budding or chain elongation. The interest here is to provide a basis for quantitative microbiology. In this context, each group of organisms is considered in terms of its physical properties, mechanism of replication, and form of growth.

2.1 Bacterial Growth

Bacteria exhibit a wide diversity in metabolic activities, but all have similar cellular structure and reproduction mechanisms. They are classed as prokaryotic organisms (BALOWS et al., 1992). The various genera of bacteria are related back to a common ancestral state (WOESE, 1987), however, through evolution, substantial structural and physiological diversity has developed. In Gram-positive cells the polysaccharide murein forms up to 30 molecular layers, while in Gram-negative cells only a single murein layer is present, and lipopolysaccharides and lipoproteins are the main constituents of the cell wall. Prokaryotes also have no nuclear membrane or other intracellular organelles.

Despite this diversity, there are several features common to many bacteria that are important to quantitative microbiology. Bacteria generally reproduce by the process of binary fission, illustrated in Fig. 1, resulting in two daughter cells of equal size. A cell grows by increasing in size, during which time the amount of each new cell component, e.g., protein, RNA, etc., is doubled and the genome is replicated. Cell division is initiated by ingrowth of the cell wall and eventual formation of a transverse septum. Cell separation proceeds by cleavage of the septum, and two identical daughter cells are formed. There is a difference, however, in how the Gram-positive and Gram-negative bacteria synthesize their cell wall material. Gram-positive bacteria synthesize new cell wall in an equatorial zone along an axis, whereas Gram-negative bacteria
synthesize cell wall by intercollation along the whole wall. Incomplete cleavage of the septa will result in chains as is the case for streptococci. A microscopic photograph is shown in Fig. 2. Delayed cleavage will result in elongated bacilli structures.

The concentration of cell components, i.e., RNA, enzymes, metabolites, etc., in each daughter will be the same as in the parent. This is true, however, only when the cells are growing in an environment that does not necessitate a change in some cell property with time. If, for instance, an environmental change calling for induction or repression of some enzyme occurs between initiation of a cell cycle and cell division, then the daughter cells will have a different level of one or more enzymes than the original parent. Some bacteria, especially members of the family Bacillaceae and some gliding bacteria (Fig. 3), have the ability to form spores to survive in adverse conditions.

**Fig. 1.** Process of binary fission and cell wall addition of a Gram-positive species of *Streptococcus* and a Gram-negative species of *Salmonella*; dark areas are old cell wall material, light areas represent newly added compounds.

**Fig. 2.** Electron micrograph of an ultrathin section of filamentous gliding Gram-negative *Herpetosiphon auranticus*. Several bacteria are sectioned longitudinally and show complete septa formation (arrow heads), bar=2 μm (LUNSDORF, GBF, 1995).

**Fig. 3.** Several fruiting bodies on a sporangiophore and sporangioles of the gliding myxobacterium *Condromyces croatus* (H. REICHENBACH, GBF).
From this discussion, it should be apparent that bacteria do not have a wide age distribution. One can talk about “old” or “young” cultures in reference to how long a flask has been incubating or in terms of how long a culture has been left standing, but the true age of a cell relates to the time since its last division.

Bacteria are generally small with a characteristic dimension of about 1 μm. They may exist as spheres or cocci, or as rod or bacilli in shape. The cells may exist as single cells or as groups or chains, e.g., diplococci (pair), streptococci (chains), staphylococci (clusters). Furthermore, many organisms contain flagella and are motile. A typical bacterial cell has a wet density (specific mass) of 1.05 to 1.1 g cm$^{-3}$ and weighs about $10^{-12}$ g as a dry particle. The density is about 1.25 g cm$^{-3}$. The actual size of a given cell will depend on its growth rate; faster growing cells are often larger. Bacteria growing on Petri dishes form colonies with a species specific in appearance. More details about industrial microorganisms can be found in DeMain and Solomon (1985).

2.2 Yeast Growth

Yeast are eukaryotic organisms which belong to the fungi. The most prominent members are baker’s yeast *S. cerevisiae*, brewer’s yeast *S. uvarum* (carlsbergensis) and the fodder yeasts *Candida utilis* and *C. tropicalis*. They are fungi that do not form asexual spores or aerial structures and exist as single cells during at least part of their vegetative growth cycle. The most common form of cell division is budding (Fig. 4), however, fission following cross-wall formation, mycelial growth by chain elongation, and branching are also observed in some yeasts (Pfaff et al., 1978). The yeasts are non-motile and non-photosynthetic. They are either oval or spherical in shape. The size of a yeast cell is dependent on the growth rate; the shorter the doubling time, the larger the cell volume. A typical cell may be about 3–7 μm of width, 5–15 μm length and has a dry cell weight (see Sect. 3.2.1) of about $10^{-11}$ g, its density being 1.05–1.1 g cm$^{-3}$ in the living state.

During growth of yeast by budding, there are several distinct events. Initially, the yeast cell undergoes a period of expansion: its volume increases. Shortly after the cell stops expanding, bud emergence occurs. During bud formation, the total volume of the mother plus daughter bud cell is constant, so that bud growth occurs as a consequence of depletion of the mother cell. The bud separates as a single, but smaller cell from the mother. Once separated, the new daughter cell and the original mother cell grow and reach the same size at the same time; thus, the daughter grows faster than the mother (Thomas et al., 1980). The mother cell will have a bud scar on its surface for each bud that has separated; these can be seen with fluorescent techniques. Unlike bacteria, mother and daughter yeast cells are different. They possess different growth rates, and their cell surface is different. It is possible to count the number of scars and to establish a cell’s age in the broth. Thus, there is a distribution of cells having different ages. While yeasts are typically single-celled, their progeny, or daughter cells, will sometimes not separate, see Fig. 5. When buds do not separate from the mother, the resulting chains of cells are called pseudomycelia.
2.3 Mycelial Growth

Molds, actinomycetes, and some yeasts under aerobic conditions, predominantly grow by the process of hypha chain elongation (WEBSTER, 1980) (e.g., elongation at the tip, also called apical growth) and branching, as shown in Fig. 6. The hypha, which is divided into individual cells, is a branching tubular structure of 2–10 μm in diameter. The intertwining strands of hyphae are called mycelium. Many fungi can form asexual spores called conidia (Fig. 7). A comprehensive overview of fungal biotechnology has been given by ELANDER and LOWE (1992).

The length of a hyphal chain depends on the growth environment. If left undisturbed, as on an agar surface, the chain can become quite long. In submerged culture, however, there are shear forces that cause hyphae fragmentation. This results in shorter, but more highly branched mycelia. In submerged cultures, the mycelia may exist along with dispersed, diffuse mycelia or may form pellets: the form of growth has an important effect on growth and product formation. A detailed discussion of mycelial growth kinetics has been presented by RIGHELATO (1979). Model-based simulations (YANG et al., 1992) (Fig. 8) have brought more insight into the process of mycelial growth.

Microorganisms respond to their environment. Cells growing within a pellet will “see” a very different environment than those growing in a more diffuse manner. A measurable bulk concentration of nutrient or product exists in the broth. However, the concentration of nutrient on the pellet surface is lower due to diffu-
sion through a stagnant liquid boundary layer and cell metabolism. The concentration is reduced further inside in the pellet as a result of metabolism, to such a degree, that, at the center of the pellet, there may be little or no nutrient. This problem is especially important when considering oxygen supply (WITTNER et al., 1986). The product concentration, on the other hand, will be higher within the pellet than outside resulting from further diffusional limitation. This is an important issue only if there is product inhibition.

Fig. 8. Simulated morphological development of the growth of a mycelium emerging from a spore: (a) $t=2.5$ h, (b) $t=4$ h, (c) $t=6$ h, (d) $t=10$ h, and (e) $t=14$ h (with kind permission from YANG et al., 1992).

The physical structure of pellets can be seen in Fig. 9. Early in a fermentation, the cells in the pellet see a nutrient-rich environment, later the pellets become more dense, and growth and metabolism occur predominantly on the periphery. As a result, cells near the pellet center become starved for one or more nutrients.

Mycelial growth leads to an age distribution of cells. Younger cells at the hyphal tip will have different metabolism than cells at or near the origin of growth. This is due to both the aging process inherent in the cell and the differing environments of young and old cells. Ideally, methods of measurement would allow the investigator to distinguish among cells of different ages. However, this is difficult to achieve, and in most cases, the performance of a mycelial population is normalized to the total amount of cell mass present.
3 Measurement and Characterization of Growth

An essential activity of fermentation technology is the quantitation of both the amount and the rate of change of microbial cell mass. This can often be achieved by direct measurement of either cell mass or number. In many cases, direct methods of measurement are not applicable, and the physiological activity, which is partially related to the amount of biomass, must be measured. Since the main point of interest for many practical applications is the activity of microorganisms, many people make a virtue of necessity. A detailed review is given in Sonnleitner et al. (1992). Unfortunately, there is no single method that is suitable for all fermentations, and a fermentation technologist needs sets of methods for growth measurement of bacteria, yeast and molds in various fermentation media. The objective of the following discussion is to present the reader with a summary of the most common methods available for microbial growth quantitation. These methods must be examined carefully for each application to be sure that they are appropriate in each case. The methods are categorized as measurement of cell number and measurement of cell mass.

From a practical point of view, it is important to classify measurements as on-line or off-line measurements. The focus lies on the latter ones, but some of the principles (e.g., turbidity, model-based estimation) are applied on-line, too. Excellent reviews on such measurement principles have been presented by Reardon and Scheper (1991) and Locher et al. (1992). In practical fermentations, sampling is done in constant time intervals. Since bioprocesses are non-linear and time-variable, optimal sampling can reduce the effort and increase the information content of the measurements (Munack, 1991).

Apart from the problems to define the amount of biomass, the mathematical definition of the specific growth rate is unequivocal. An increase (or decrease) in the amount of biomass $X$ or cell number $N$ is assumed to be proportional to the total amount actually present, thus yielding

\[ \dot{X}(t) = \mu(t) \cdot X(t) \quad (1a) \]

where the (arbitrarily time-dependent) factor $\mu$ is called the specific growth rate. The definition makes sense especially if each part and fraction of the biomass has the equal capacity to grow. This lumped definition of growth rate does not imply any assumptions about the status of individual cells, where some may proliferate, while others may show lysis.

The definition of the doubling time $t_d$ of a cell population, where

\[ N(t + t_d) = 2 \cdot N(t) \quad (1b) \]

is commonly used to describe growth in terms of cell number or cell mass. Integration of Eq. (1a) (see Sect. 6.1 and Eq. 12) and application to Eq. (1b) gives

\[ t_d = \ln 2/\mu \quad (1c) \]

which relates the number- or mass-based doubling time to the specific growth rate.

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**Fig. 9.** Photograph showing a pellet during a fermentation of *Penicillium chrysogenum.*
The number-based definition is less useful in cases of segregated biomass by age or activity distribution or even of different organisms. Fig. 10 shows the development of a population of recombinant cells, where different growth rates for different cell types apply. If only a couple of single cells are investigated according to cell number where each cell fission changes the population remarkably, then statistical definitions for growth are implemented.

3.1 Measurement of Cell Number

Cell number is, at least in the case of singly suspended cells, a biologically appropriate defined value to measure an amount of biological material. Conceptual and practical problems arise if formation of cell aggregates occur. These problems include how to define the point where a growing bud of a yeast cell becomes an individual cell, how to distinguish between a dividing cell and two just divided cells in a chain of bacteria or how to count fungal cells in a pellet, especially if fast formation of biological material at the tip of the hyphae and lysis in elder parts is observed. The concept of viability plays an important role. The definition of a single cell to be counted involves not only particles surrounded by a cell membrane, but also qualities representing life directly, such as physiological activity and capability to divide measured as capability of colony formation. The techniques of cell counting presented here provide the reader with an account of the present possibilities. However, for practical application a textbook for microbiological methods should be consulted (e.g., Gerhardt et al., 1981).

3.1.1 Direct Microscopic Count

A rapid method for counting the total number of cells is direct microscopic count utilizing a counting chamber, such as the Petroff–Hausser slide. It consists of a microscopic slide with a depression of known depth and a cover slip marked with a grid of known area. With a phase contrast microscope, a sufficiently high number of cells (typically 500 and more) is counted under an appropriate number of grid squares. The cell density is then calculated from the average cell number per square divided by the corresponding fluid volume beneath the square. The resulting data indicate the total number of cells, but do not quantitate the number of viable cells unless a viable stain such as methylene blue is used. This stain is oxidized to a colorless form by cells capable of respiring, a trait usually associated with viability although it is possible for non-dividing cells...
to still respire. Dead or non-respiring cells will stain blue.

The use of direct microscopic counts depends on the ability to distinguish and count individual cells and is inapplicable to mycelial or chain-forming organisms. Actively mobile bacteria can be sedimented by adding 0.5% of formalin to the cell suspension; this facilitates counting.

Measurement of cell number in counting chambers is primarily designed for microbial cultures which usually must be substantially diluted prior to counting. In environmental samples, such as river and lake water, the cell number usually is too low for this procedure. The most widely accepted technique to count these microorganisms is membrane filtration combined with epifluorescence microscopy which makes detection of bacteria easier and allows discrimination among living cells, dead cells and detritus to some extent. The preparation of the filter (preferably polycarbonate) involves drying, staining with a fluorochrome dye such as acridine orange, decoloration and embedding in immersion oil.

3.1.2 Viable Plate Counts

The most common method for determining the number of viable cells is to perform a viable plate count. This technique is based on the principle that a population of cells can be spread across a solid nutrient medium such that each cell is separated from the other and, upon division, will form a distinct colony in which all progeny are derived from a single parent forming what is called a clone; this process is called cloning. The most common solid medium is agar. The nutrient medium is prepared by mixing the required growth nutrient with typically 1.5–2.5% agar and, after heating to above 100 °C, solidifies when cooled below about 45 °C. This medium is commonly poured into Petri dishes. Culture samples need to be diluted such that 30 to 300 cells are applied to one 100 mm diameter Petri dish. More than 300 colonies (about 25 mm² of plate area per colony) will lead to overcrowding and difficulty when counting the colonies.

Considerable care is needed when interpreting the results of a viable plate count. This technique measures the number of colony-forming units (CFU) that can form in the environment of the plates. Cells that agglomerate, form chains or hyphae, cannot be counted by this technique, for colonies may evolve from more than a single cell. The solid surface of agar is a different environment than submerged culture, and viability (percent of total organism that will divide and grow) on a plate may be very different than in liquid broth. Motile bacteria will swarm over the plate surface and not form distinct colonies, particularly if the agar surface was not properly dried.

Plate counting requires little capital expense, but significant labor is involved in preparing and counting plates. After plating, the cultures are typically incubated for 24–72 h to allow the colonies to form.

A modified version of the plate count is the slide culture technique. This technique, described by Postgate et al. (1969), allows one to place a culture sample onto a small agar medium plate mounted on a microscopic slide. After incubation for 2–4 doubling times, microcolonies will form from viable cells. With the microscope, it is possible to do both a total and viable cell count.

A convenient method to do total bacterial counts in dilute suspensions is to filter a known volume sample with a membrane filter (pore size 0.22 μm) such that the cells are trapped on the filter surface. The filter then is laid on top of a suitable nutrient agar medium and incubated until colonies appear; knowing the volume of the sample filtered, the original concentration of viable organisms can be calculated. With a microscope, both total and viable cell counts can be made; often staining procedures and side lighting aid counting.

All the methods described also apply to viable counts in natural water and soils. However, it must be kept in mind that these samples contain a highly diverse bacterial population of which only a small fraction will grow on the medium and under the conditions provided. Only in highly polluted environments will the viable counts approach the numbers directly counted on membrane filters.