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Products from Micro-organisms

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Introduction

Micro-organisms are capable of growing on a wide range of substrates and can produce a remarkable spectrum of products. The relatively recent advent of *in vitro* genetic manipulation has extended the range of products that may be produced by micro-organisms and has provided new methods for increasing the yields of existing ones. The commercial exploitation of the biochemical diversity of micro-organisms has resulted in the development of the fermentation industry and the techniques of genetic manipulation have given this well-established industry the opportunity to develop new processes and to improve existing ones. The term 'fermentation' is derived from the Latin verb *fervere*, to boil, which describes the appearance of the action of yeast on extracts of fruit or malted grain during the production of alcoholic beverages. However, 'fermentation' is interpreted differently by microbiologists and biochemists. To a microbiologist the word means any process for the production of a product by the mass culture of micro-organisms. To a biochemist, however, the word means 'an energy-generating process in which organic compounds act as both electron donors and acceptors', that is, an anaerobic process where energy is produced without the participation of oxygen or other inorganic electron acceptors. In this chapter 'fermentation' is used in its broader, microbiological context.

Microbial Growth

The growth of a micro-organism may result in the production of a range of

metabolites but to produce a particular metabolite the desired organism must be grown under precise cultural conditions at a particular growth rate. If a micro-organism is introduced into a nutrient medium which supports its growth, the inoculated culture will pass through a number of stages and the system is termed batch culture. Initially, growth does not occur and this period is referred to as the lag phase and may be considered a period of adaptation. Following an interval during which the growth rate of the cells gradually increases, the cells grow at a constant, maximum rate and this period is referred to as the log, or exponential phase, which may be described by the equation:

$$\frac{dx}{dt} = \mu x \qquad (1)$$

where x is the cell concentration (mg cm^{-3})

t is the time of incubation (h) and

μ is the specific growth rate (h^{-1}).

On integration equation 1 gives

$$x_t = x_o e^{\mu t} \qquad (2)$$

where x_o is the cell concentration at time zero and

x_t is the cell concentration after a time interval, t hours.

Thus, a plot of the natural logarithm of the cell concentration against time gives a straight line, the slope of which equals the specific growth rate which is the maximum for the prevailing conditions and is thus described as the maximum specific growth rate, or μ_{max}. Equations 1 and 2 ignore the facts that growth results in the depletion of nutrients and the accumulation of toxic by-products and thus predict that growth continues indefinitely. However, in reality, as substrate (nutrient) is exhausted and toxic products accumulate, the growth rate of the cells deviates from the maximum and eventually growth ceases and the culture enters the stationary phase. After a further period of time, the culture enters the death phase and the number of viable cells

declines. This classic description of microbial growth is illustrated in Figure 1 and it should be remembered that this description refers to the behaviour of both unicellular and mycelial (filamentous) micro-organisms in batch culture, the growth of the latter resulting in the exponential addition of viable biomass to the mycelial body rather than the production of separate, discrete unicells.

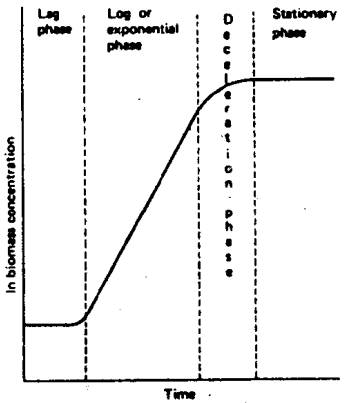

Figure 1. Growth of a 'typical' micro-organism under batch culture conditions. (Reproduced with permission from Pergamon Press, Stanbury, P.F. and Whitaker, A., 1984, Principles of Fermentation Technology)

As already stated, the cessation of growth in a batch culture may be due to the exhaustion of a nutrient component or the accumulation of a toxic product. However, provided that the growth medium is designed such that growth is limited by the availability of a medium component, growth may be extended by addition of an aliquot of fresh medium to the vessel. If the fresh medium is added continuously, at an appropriate rate, and the culture vessel is fitted with an overflow device, such that culture is displaced by the incoming fresh medium, a continuous culture may be established. The growth of the cells in a continuous culture of this type is controlled by the availability of the growth-limiting chemical component of the medium and, thus, the system is described as a chemostat. In this system a steady state is eventually achieved and the loss of biomass via the overflow is replaced by cell growth. The flow of medium through the system is described by the term dilution rate, D, which is equal to the rate of addition of medium divided by the working volume of the culture vessel. The balance between growth of cells and their loss from the

system may be described as

$$\frac{dx}{dt} = \text{growth} - \text{output}$$

or

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

under steady state conditions,

$$\frac{dx}{dt} = 0$$

therefore, $\mu x = Dx$ and $\mu = D$.

Hence, the growth rate of the organisms is controlled by the dilution rate which is an experimental variable. It will be recalled that under batch culture conditions an organism will grow at its maximum specific growth rate and, therefore, it is obvious that a continuous culture may be operated only at dilution rates below the maximum specific growth rate. Thus, within certain limits, the dilution rate may be used to control the growth rate of a chemostat culture.

The mechanism underlying the controlling effect of the dilution rate is essentially the relationship between μ, specific growth rate, and s, the limiting substrate concentration in the chemostat, demonstrated by Monod[1] in 1942:

$$\mu = \mu_{max} \frac{s}{K_s + s} \qquad (3)$$

where K_s is the utilisation or saturation constant which is numerically equal to the substrate concentration when μ is half μ_{max}.

At steady state $\mu = D$

Therefore,

$$D = \mu_{max} \frac{\bar{s}}{K_s + \bar{s}}$$

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

and

$$\bar{s} = \frac{K_s D}{\mu_{max} - D} \qquad (4)$$

Equation 4 predicts that the substrate concentration is determined by the dilution rate. In effect, this occurs by growth of the cells depleting the substrate to a concentration that supports the growth rate equal to the dilution rate. If substrate is depleted below the level that supports the growth rate dictated by the dilution rate the following sequence of events takes place:

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 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.

Thus, 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 growth rates.

Fed-batch culture is a system which may be considered to be intermediate between batch and continuous processes. The term fed-batch is used to describe batch cultures which are fed continuously, or sequentially, with fresh medium without the removal of culture fluid. Thus, the volume of a fed-batch culture

increases with time. Pirt[2] (1975) described the kinetics of such a system as follows:

If the growth of an organism were limited by the concentration of one substrate in the medium the biomass at stationary phase, x_{max}, would be described by the equation

$$x_{max} = YS_R$$

where Y is the yield factor and is equal to the mass of cells produced per gram of substrate and

S_R is the initial concentration of the growth limiting substrate.

If fresh medium were to be added to the vessel at a dilution rate less than μ_{max} then virtually all the substrate would be consumed as it entered the system:

$$F\ S_R = \mu \frac{X}{Y}$$

where F is the flow rate and

X is the total biomass in the vessel, ie the cell concentration multiplied by the culture volume.

Although the total biomass (X) in the vessel increases with time the concentration of cells, x, remains virtually constant, thus $\frac{dx}{dt} = 0$, $\mu = D$. Such a system is then described as quasi-steady state. As time progresses and the volume of culture increases the dilution rate decreases. Thus, the value of D is given by the expression

$$D = \frac{F}{V_o + Ft}$$

where F is the flow rate

V_o is the initial culture volume and

t is time

Monod[1] kinetics predict that as D falls residual substrate should also decrease resulting in an increase in biomass. However, over the range of growth rates operating the increase in biomass should be insignificant. The major difference between the steady state of the chemostat and the quasi-steady state of a fed-batch culture is that in a chemostat, D is constant whereas in a fed-batch system D decreases with time. The dilution rate in a fed-batch system may be kept constant by increasing, exponentially, the flow rate using a computer control system.

Fermentation Processes

Stanbury and Whitaker[3] classified microbial fermentations into four major groups

i) Those that produce microbial cells (biomass) as the product

ii) Those that produce microbial enzymes

iii) Those that produce microbial metabolites

iv) Those that modify a compound which is added to the fermentation - the transformation processes.

Microbial Biomass. Microbial biomass is produced commercially as single cell protein (SCP) for human food or animal feed and as viable yeast cells to be used in the baking industry. The industrial production of bakers' yeast started in the early 1900's and yeast biomass was used as human food in Germany during the First World War. However, the development of large scale processes for the production of microbial biomass as a source of commercial protein began in earnest in the late 1960's. Several of the processes investigated did not come to fruition due to political and economic problems but the establishment of the ICI pruteen process for the production of bacterial SCP was a milestone in the development of the fermentation industry[4]. This process utilises

continuous culture on an enormous scale (1500 m^3) and is an excellent example of the application of good engineering to the design of a microbiological process. The economics of the production of SCP as animal feed are still marginal but ICI are collaborating with Rank Hovis and MacDougalls on a process for the production of fungal biomass to be used as human food and the economics of such a system should prove more attractive.

Microbial Metabolites. The kinetic description of batch culture may be rather misleading when considering the product forming capacity of the culture during the various phases, for, although the metabolism of stationary-phase cells is considerably different from that of logarithmic ones, it is by no means stationary. Bu'Lock et al.[5] proposed a descriptive terminology of the behaviour of microbial cells which considered the type of metabolism rather than the kinetics of growth. The term trophophase was suggested to describe the log or exponential phase of a culture during which the sole products of metabolism are either essential to growth such as amino acids, nucleotides, proteins, nucleic acids, lipids, carbohydrates, etc or are the by-products of energy yielding metabolism such as ethanol, acetone and butanol. The metabolites produced during the trophophase are referred to as primary metabolites. Some examples of primary metabolites of commercial importance are listed in Table 1.

Table 1. Some examples of microbial primary metabolites and their commercial significance

Primary metabolite	Producing organism	Commercial significance
Ethanol	Saccharomyces cerevisiae	'Active ingredient' in alcoholic beverages
Citric acid	Aspergillus niger	Various uses in the food industry
Acetone and butanol	Clostridium acetobutyricum	Solvents
Glutamic acid	Corynebacterium glutamicum	Flavour enhancer
Lysine	Corynebacterium glutamicum	Feed additive
Polysaccharides	Xanthomonas spp	Applications in the food industry; enhanced oil recovery
Fe^{+++}	Thiobacillus and Sulfolobus	Ore leaching

The term idiophase was suggested to describe the phase of a culture during which products other than primary metabolites are synthesised, products which do not have an obvious role in cell metabolism. The metabolites produced during the idiophase are referred to as the secondary metabolites. The inter-relationships between primary and secondary metabolism are illustrated in Figure 2, from which it may be seen that secondary metabolites tend to be synthesised from the intermediates and end products of primary metalbolism. Although the primary metabolic routes shown in Figure 2 are common to the vast majority of micro-organisms, each secondary metabolite would be synthesised by very few microbial taxa. Also, not all microbial taxa undergo secondary metabolism; it is a common feature of the filamentous fungi and bacteria and the sporing bacteria but it is not, for example, a feature of the Enterobacteriaceae. Thus, although the taxonomic distribution of secondary metabolism is far more limited than that of primary metabolism, the range of secondary products produced is enormous.

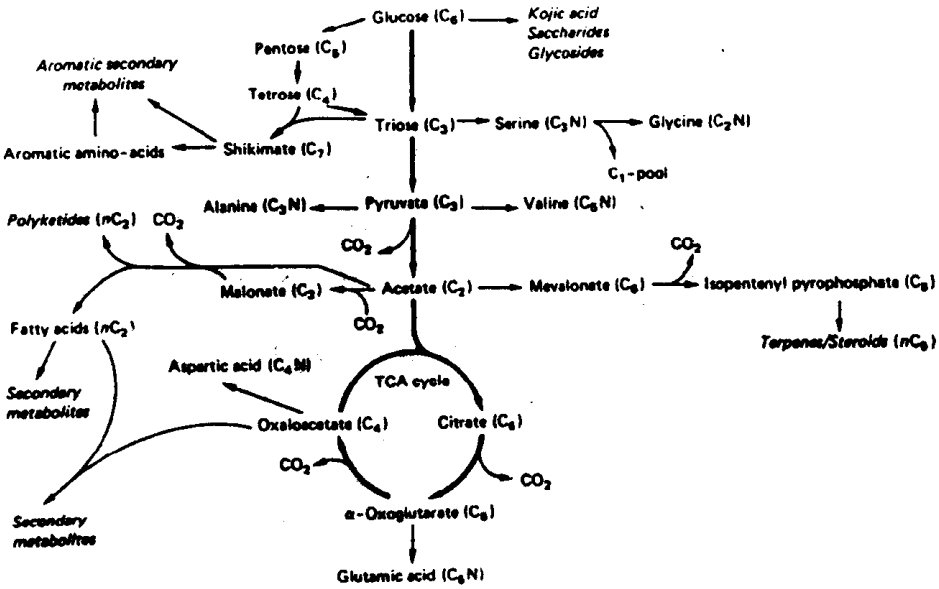

Figure 2. The inter-relationships between primary and secondary metabolism (Reproduced with permission from Academic Press, Turner, W.B., 1971, Fungal Metabolites)

At first sight it may seem anomalous that micro-organisms produce compounds which do not appear to have any metabolic function and are certainly not by-products of catabolism as are, for example, ethanol and acetone. However, many secondary metabolites exhibit antimicrobial properties and, therefore, may be involved in competition in the natural environment[6]; others have, since their discovery in idiophase cultures, been demonstrated to be produced during the trophophase where, it has been claimed, they act in some form of metabolic control[7]. Although the physiological role of secondary metabolism continues to be the subject of considerable debate its relevance to the fermentation industry is the commercial significance of the secondary metabolites. Table 2 summarises some of the industrially important groups of secondary metabolites.

Table 2. Some examples of microbial secondary metabolites and their commercial significance

Secondary metabolite	Commercial significance
Penicillin	Antibiotic
Cephalosporin	Antibiotic
Tetracyclines	Antibiotic
Streptomycin	Antibiotic
Griseofulvin	Antibiotic (anti-fungal)
Actinomycin	Antitumour
Pepstatin	Treatment of ulcers
Cyclosporin A	Immunosuppressant
Krestin	Cancer treatment
Bestatin	Cancer treatment
Gibberellin	Plant growth regulator

The production of microbial metabolites may be achieved in continuous, as well as batch systems. The chronological separation of trophophase and idiophase in batch culture may be studied in continuous culture in terms of dilution rate[8-10]. Secondary metabolism will occur at relatively low dilution rates (growth rates) and, therefore, it should be remembered that secondary metabolism is a property of slow-growing, as well as stationary, cells. The fact that secondary metabolites are produced by slow-growing organisms in continuous culture indicates that primary metabolism is continuing in idiophase-type cells and that secondary metabolism is not switched on to remove an accumulation of metabolites synthesised entirely in a different phase, but that synthesis of the primary metabolic precursors continues through the period

of secondary biosynthesis.

The control of the onset of secondary metabolism has been studied extensively in batch culture and, to a lesser extent, in continuous culture. The outcome of this work is that a considerable amount of information is available on the changes occurring in the medium at the onset of secondary metabolism but relatively little is known of the control of the process at the DNA level. Primary metabolic precursors of secondary metabolites have been demonstrated to induce their formation, for example tryptophan in alkaloid[11] biosynthesis and methionine in cephalosporin biosynthesis[12]. On the other hand, medium components have been demonstrated to repress secondary metabolism, the earliest observation being that of Saltero and Johnson[13] in 1953 of the repressing effect of glucose on benzyl penicillin formation. Carbon sources which support high growth rates tend to support poor secondary metabolism and Table 3 cites some examples of this situation. Phosphate and nitrogen sources have also been implicated in the repression of secondary metabolism, as exemplified in Table 3. Therefore, it is essential that repressing nutrients should be avoided in media to be used for the industrial production of secondary metabolites or that the mode of operation of the fermentation maintains the potentially repressing components at sub-repressing levels, as discussed in a later section of this chapter.

Table 3. Some examples of the repression of secondary metabolism by medium components

Medium component	Repressed secondary metabolite
Glucose	Penicillin[13-14]
Glucose	Chloramphenicol[15]
Glucose	Actinomycin[30]
Glucose	Neomycin[31]
Glucose	Streptomycin[32]
Glucose	Cephalosporin[33]
Phosphate	Candicidin[16]
Phosphate	Streptomycin[18]
Phosphate	Tetracycline[65]
Nitrogen source	Penicillin[17]

As mentioned in the introduction, the advent of recombinant DNA technology has extended the potential range of products that may be produced by micro-organisms. Microbial cells may be endowed with the ability to produce compounds normally associated with higher cells and such products may form the basis of new fermentation processes, for example the synthesis of interferon, insulin and renin. It seems to be a widely held opinion that the methods of genetic manipulation will revolutionise the fermentation industry and give rise to a large number of new processes. However, as pointed out by Stanbury and Whitaker[3], the exploitation of these advances depends upon the technology of mass cell culture which has evolved from the yeast and solvent fermentations, via the antibiotic fermentations, to the large scale continuous biomass processes. Indeed, in a recent appraisal[19] of the fermentation market in the United States it is claimed that, although the ultimate fruits of genetic engineering are impossible to forecast, the older biotechnology of standard fermentation will remain a highly viable, and predictably growing, method of industrial production. A similar report[20] on the fermentation market in Europe is in press.

Microbial Enzymes. The major commercial utilisation of enzymes is in the food and beverage industries[21] although enzymes do have considerable application in clinical and analytical situations as well as their use in washing powders. Enzymes may be produced from animals and plants as well as microbial sources but the production by microbial fermentation is the most economic and convenient method. Furthermore, it is now possible to engineer microbial cells to produce animal or plant enzymes, for example the production of renin by *E. coli*. Most enzymes are synthesised in the logarithmic phase of batch culture and may, therefore, be considered as primary metabolites. However, some, for example the amylases of *Bacillus stearothermophilus*[22], are produced by idiophase type cultures and may be considered as equivalent to secondary metabolites.