CHAPTER 1

# Fermentation Technology

PETER F. STANBURY

## 1 INTRODUCTION

Microorganisms 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 microorganisms and has provided new methods for increasing the yields of existing ones. The commercial exploitation of the biochemical diversity of microorganisms 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 microorganisms. 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.

## 2 MICROBIAL GROWTH

The growth of a microorganism 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 microorganism is introduced into a nutrient medium that 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

$$dx/dt = \mu x \tag{1}$$

where $x$ is the cell concentration (mg $ml^{-1}$), $t$ is the time of incubation (h), and $\mu$ the specific growth rate ($h^{-1}$). On integration equation (1) gives

$$x_t = x_0 e^{\mu t} \tag{2}$$

where $x_0$ is the cell concentration at time zero and $x_t$ is the cell concentration after a time interval, $t$ h.

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. The specific growth rate during the exponential phase is the maximum for the prevailing conditions and is described as the maximum specific growth rate, or $\mu_{max}$. 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 representation of microbial growth is illustrated in Figure 1. It should be remembered that this description refers to the behaviour of both unicellular and mycelial (filamentous) 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.

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,

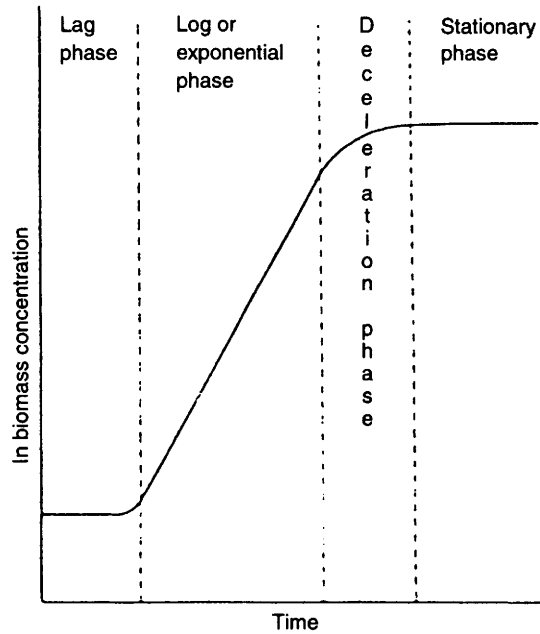


**Figure 1** *Growth of a 'typical' microorganism under batch culture conditions* (Reproduced with permission from P. F. Stanbury, A. Whitaker and S. J. Hall, 'Principles of Fermentation Technology', Pergamon Press, Oxford, 1995)

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

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

or

$$dx/dt = \mu x - Dx$$

Under steady-state conditions,

$$dx/dt = 0$$

and, 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 $\mu$, specific growth rate, and $s$, the limiting substrate concentration in the chemostat, demonstrated by Monod[1] in 1942:

$$\mu = \mu_{max} s/(K_s + s) \tag{3}$$

where $K_s$ is the utilization or saturation constant, which is numerically equal to the substrate concentration when $\mu$ is half $\mu_{max}$. At steady-state, $\mu = D$, and, therefore,

$$D = \mu_{max} \bar{s}/(K_s + \bar{s})$$

Where $\bar{s}$ is the steady-state concentration of substrate in the chemostat, and

$$\bar{s} = K_s D/(\mu_{max} - D) \tag{4}$$

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 that 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.

[1] J. Monod, 'Recherches sur les Croissances des Cultures Bacteriennes', Herman and Cie, Paris, 1942.

(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.

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

Fed-batch culture is a system that may be considered to be intermediate between batch and continuous processes. The term fed-batch is used to describe batch cultures that 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] 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 consumed 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 $\mu_{max}$ then virtually all the substrate would be consumed as it entered the system:

$$FS_R = \mu(X/Y)$$

where $F$ is the flow rate and $X$ is the total biomass in the vessel, *i.e.* 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 $dx/dt = 0$ and $\mu = D$. 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

$$D = F/(V_0 + F_t)$$

where $F$ is the flow rate, $V_0$ is the initial culture volume, and $t$ is time. Monod[1] kinetics predict that as $D$ falls residual substrate concentration

[2] S. J. Pirt, 'Principles of Microbe and Cell Cultivation', Blackwell, Oxford, 1975.

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 $D$ (hence, $\mu$) is constant whereas in a fed-batch system $D$ (hence, $\mu$) 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.

## 3 APPLICATIONS OF FERMENTATION

Microbial fermentations may be classified into the following major groups:[3]

(i) Those that produce microbial cells (biomass) as the product.
(ii) Those that produce microbial metabolites.
(iii) Those that produce microbial enzymes.
(iv) Those that modify a compound which is added to the fermentation – the transformation processes.
(v) Those that produce recombinant products.

### 3.1 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 1900s 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 1960s. Several of the processes investigated did not come to fruition owing to political and economic problems but the establishment of the ICI Pruteen process for the production of bacterial SCP for animal feed was a milestone in the development of the fermentation industry.[4] This process utilized 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. However, the economics of the production of SCP as animal feed were marginal, which eventually led to the discontinuation of the

[3] P. F. Stanbury, A. Whitaker and S. J. Hall, 'Principles of Fermentation Technology', 2nd Edn, Pergamon Press, Oxford, 1995.
[4] D. H. Sharp, 'Bioprotein Manufacture—A Critical Assessment', Ellis Horwood, Chichester, 1989, Chapter 4, p. 53.

Pruteen process. The technical expertise gained from the Pruteen process assisted ICI in collaborating with Rank Hovis MacDougall on a process for the production of fungal biomass to be used as human food.[5] A continuous fermentation process for the production of *Fusarium graminearum* biomass (marketed as Quorn®) was developed utilizing a 40 $m^3$ air-lift fermenter. This process was based on sound economics and has proved to be a major economic success.

## 3.2 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.*[6] 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.

Bu'Lock *et al.* suggested the term 'idiophase' to describe the phase of a culture during which products other than primary metabolites are synthesized, 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 interrelationships between primary and secondary metabolism are illustrated in Figure 2, from which it may be seen that secondary metabolites tend to be synthesized from the intermediates and end-products of primary metabolism. Although the primary metabolic routes shown in Figure 2 are common to the vast majority of microorganisms, each secondary metabolite would be synthesized 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

[5] A. P. J. Trinci, *Mycol. Res.*, 1992, **96**, 1.
[6] J. D. Bu'Lock, D. Hamilton, M. A. Hulme, A. J. Powell, D. Shepherd, H. M. Smalley and G. N. Smith, *Can. J. Microbiol.*, 1965, **11**, 765.

**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 food industry |
| Glutamic acid | *Corynebacterium glutamicum* | Flavour enhancer |
| Lysine | *Corynebacterium glutamicum* | Feed additive |
| Polysaccharides | *Xanthamonas* spp. | Applications in food industry; enhanced oil recovery |

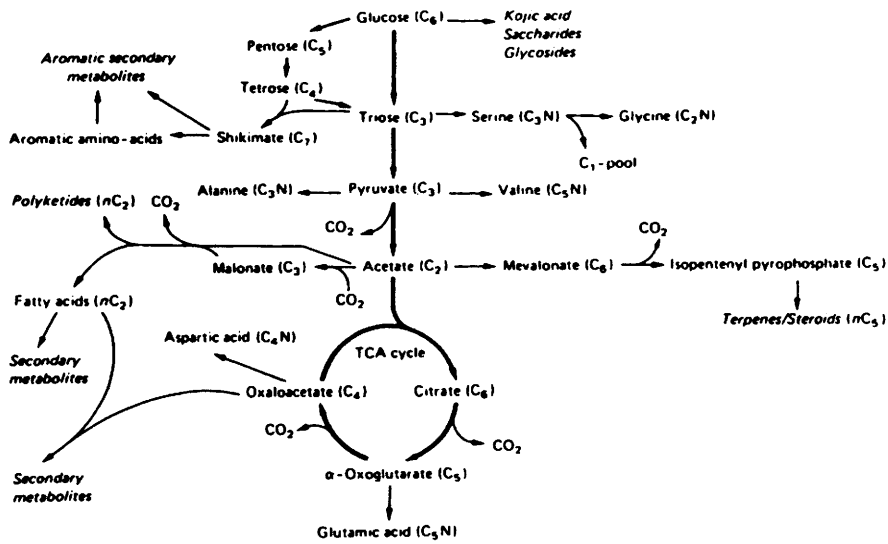


**Figure 2** *The inter-relationships between primary and secondary metabolism* (Reproduced with permission from W. B. Turner, 'Fungal Metabolites', Academic Press, 1971)

taxonomic distribution of secondary metabolism is far more limited than that of primary metabolism, the range of secondary products produced is enormous. The classification of microbial products into secondary and primary metabolites should be considered as a convenient, but in some cases, artificial system. To quote Bushell,[7] the classification should not be allowed to act as a conceptual straitjacket, forcing the reader to consider all products as either primary or secondary metabolites. It is sometimes difficult to categorize a product as primary or secondary, and the kinetics

[7] M. E. Bushell, in 'Principles of Biotechnology', ed. A. Wiseman, Chapman and Hall, New York, 1988, p. 5.

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

| *Secondary metabolite* | *Commercial significance* |
|---|---|
| Penicillin | Antibiotic |
| Cephalosporin | Antibiotic |
| Streptomycin | Antibiotic |
| Griseofulvin | Antibiotic (anti-fungal) |
| Pepstatin | Treatment of ulcers |
| Cyclosporin A | Immunosuppressant |
| Gibberellin | Plant growth regulator |
| Lovastatin | Cholesterol synthesis inhibitor |

of production of certain compounds may change, depending on the growth conditions employed.

At first sight it may seem anomalous that microorganisms 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;[8] 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.[9] 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 summarizes some of the industrially important groups of secondary metabolites.

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.[10–12] 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

[8] A. L. Demain, *Search*, 1980, **11**, 148.
[9] I. M. Campbell, *Adv. Microb. Physiol.*, 1984, **25**, 2.
[10] S. J. Pirt, *Chem. Ind. (London)*, May 1968, 601.
[11] S. J. Pirt and R. C. Righelato, *Appl. Microbiol.*, 1967, **15**, 1284.
[12] L. H. Christensen, C. M. Henriksen, J. Nielson, J. Villadsen and M. Egel-Mitani, *J. Biotechnol.*, **42**, 95.

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

| *Medium component* | *Repressed secondary metabolite* |
|---|---|
| Glucose | Penicillin |
| Glucose | Actinomycin |
| Glucose | Neomycin |
| Glucose | Streptomycin |
| Phosphate | Candicidin |
| Phosphate | Streptomycin |
| Phosphate | Tetracycline |

that primary metabolism is continuing in idiophase-type cells. Thus, secondary metabolism is not switched on to remove an accumulation of metabolites synthesized entirely in a different phase; 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 interrelationships between the changes occurring in the medium and the cells at the onset of secondary metabolism and the control of the process. Primary metabolic precursors of secondary metabolites have been demonstrated to induce secondary metabolism, for example, tryptophan in alkaloid[13] biosynthesis and methionine in cephalosporin biosynthesis.[14] On the other hand, medium components have been demonstrated to repress secondary metabolism, the earliest observation being that of Saltero and Johnson[15] in 1953 of the repressing effect of glucose on benzyl penicillin formation. Carbon sources that support high growth rates tend to support poor secondary metabolism and Table 3 cites some examples of this situation. Phosphate 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.

[13] J. F. Robers and H. G. Floss, *J. Pharmacol. Sci.*, 1970, **59**, 702.
[14] K. Komatsu, M. Mizumo and R. Kodaira, *J. Antibiot.*, **28**, 881.
[15] F. V. Saltero and M. I. Johnson, *Appl. Microbiol.*, 1953, **1**, 2.

### 3.3 Microbial Enzymes

The major commercial utilization of microbial enzymes is in the food and beverage industries[16] although enzymes do have considerable application in clinical and analytical situations, as well as their use in washing powders. Most enzymes are synthesized in the logarithmic phase of batch culture and may, therefore, be considered as primary metabolites. However, some, for example the amylases of *Bacillus stearothermophilus*,[17] are produced by idiophase cultures and may be considered as equivalent to secondary metabolites. 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, as discussed in Section 3.5.

### 3.4 Transformation Processes

As well as the use of microorganisms to produce biomass and microbial products, microbial cells may be used to catalyse the conversion of a compound into a structurally similar, but financially more valuable, compound. Such fermentations are termed transformation processes, biotransformations, or bioconversions. Although the production of vinegar is the oldest and most well-established transformation process (the conversion of ethanol into acetic acid), the majority of these processes involve the production of high-value compounds. Because microorganisms can behave as chiral catalysts with high regio- and stereospecificity, microbial processes are more specific than purely chemical ones and make possible the addition, removal, or modification of functional groups at specific sites on a complex molecule without the use of chemical protection. The reactions that may be catalysed include oxidation, dehydrogenation, hydroxylation, dehydration and condensation, decarboxylation, deamination, amination, and isomerization. The anomaly of the transformation process is that a large biomass has to be produced to catalyse, perhaps, a single reaction. The logical development of these processes is to perform the reaction using the purified enzyme or the enzyme attached to an immobile support. However, enzymes work more effectively within their microbial cells, especially if co-factors such as reduced pyridine nucleotide need to be

[16] D. J. Jeenes, D. A. MacKenzie, I. N. Roberts and D. B. Archer, in 'Biotechnology and Genetic Engineering Reviews', ed. M. P. Tombs, Intercept, Andover, 1991, Vol. 9, Chapter 9, p. 327.
[17] A. B. Manning and L. L. Campbell, *J. Biol. Chem.*, 1961, **236**, 2951.

regenerated. A compromise is to employ resting cells as catalysts, which may be suspended in a medium not supporting growth or attached to an immobile support. The reader is referred to Goodhue *et al.*[18] for a detailed review of transformation processes.

### 3.5 Recombinant Products

The advent of recombinant DNA technology has extended the range of potential microbial fermentation products. It is possible to introduce genes from higher organisms into microbial cells such that the recipient cells are capable of synthesizing foreign (or heterologous) proteins. Examples of the hosts for such foreign genes include *Escherichia coli*, *Saccharomyces cerevisiae* and other yeasts as well as filamentous fungi such as *Aspergillus niger* var. *awamori*. Products produced in such genetically manipulated organisms include interferon, insulin, human serum albumin, factor VIII and factor IX, epidermal growth factor, bovine somatostatin and bovine chymosin. Important factors in the design of these processes include the secretion of the product, minimization of the degradation of the product, and the control of the onset of synthesis during the fermentation, as well as maximizing the expression of the foreign gene. These aspects are considered in detail in references 19, 20 and 21.

## 4 THE FERMENTATION PROCESS

Figure 3 illustrates the component parts of a generalized fermentation process. Although the central component of the system is obviously the fermenter itself, in which the organism is grown under conditions optimum for product formation, one must not lose sight of operations upstream and downstream of the fermenter. Before the fermentation is started the medium must be formulated and sterilized, the fermenter sterilized, and a starter culture must be available in sufficient quantity and in the correct physiological state to inoculate the production fermenter. Downstream of the fermenter the product has to be purified and further processed and the effluents produced by the process have to be treated.

[18] C. T. Goodhue, J. P. Rosazza and G. P. Peruzzutti, in 'Manual of Industrial Microbiology and Biotechnology', ed. A. L. Demain and A. Solomons, American Society for Microbiology, Washington, DC, 1986, p. 97.
[19] J. R. Harris, 'Protein Production by Biotechnology', Elsevier, London, 1990.
[20] A. Wiseman, 'Genetically-engineered Proteins and Enzymes from Yeast: Production and Control', Ellis Horwood, Chichester, 1991.
[21] R. C. Hockney, *Trends in Biotechnology*, **12**, 456.

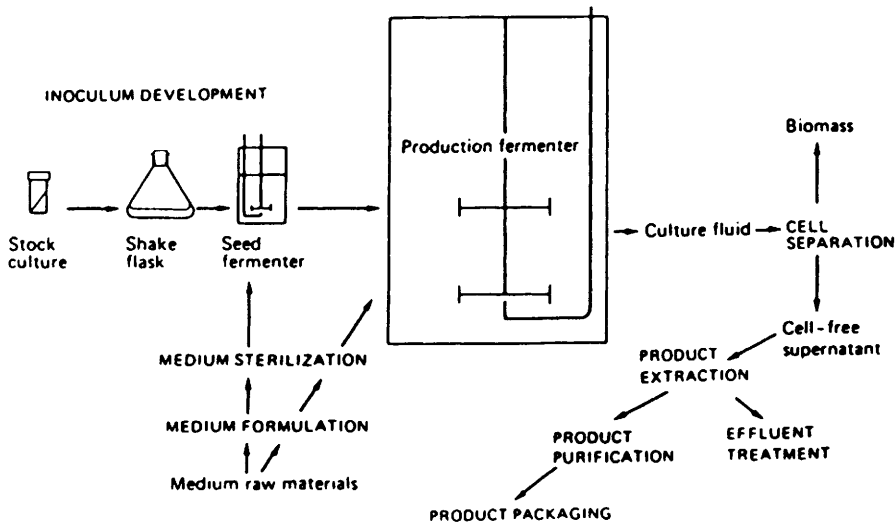


**Figure 3** *A generalized, schematic representation of a fermentation process* (Reproduced with permission from P. F. Stanbury, A. Whitaker and S. J. Hall, 'Principles of Fermentation Technology', Pergamon Press, Oxford, 1995)

## 4.1 The Mode of Operation of Fermentation Processes

As discussed earlier, microorganisms may be grown in batch, fed-batch, or continuous culture, and continuous culture offers the most control over the growth of the cells. However, the commercial adoption of continuous culture is confined to the production of biomass and, to a limited extent, the production of potable and industrial alcohol. The superiority of continuous culture for biomass production is overwhelming, as may be seen from the following account, but for other microbial products the disadvantages of the system outweigh the improved process control which the technique offers.

Productivity in batch culture may be described by the equation[3]

$$R_{\text{batch}} = (x_{\max} - x_0)/(t_i + t_{ii}) \tag{5}$$

where $R_{\text{batch}}$ is the output of the culture in terms of biomass concentration per hour, $x_{\max}$ is the maximum cell concentration achieved at stationary phase, $x_0$ is the initial cell concentration at inoculation, $t_i$, is the time during which the organism grows at $\mu_{\max}$ and $t_{ii}$ is the time during which the organism is not growing at $\mu_{\max}$ and includes the lag phase, the deceleration phase, and the periods of batching, sterilizing and harvesting.

The productivity[3] of a continuous culture may be represented as

$$R_{cont} = D\bar{x}(1 - t_{iii}/T) \tag{6}$$

where $R_{cont}$ is the output of the culture in terms of cell concentration per hour, $t_{iii}$ is the time period prior to the establishment of a steady-state and includes time for vessel preparation, sterilization and operation in batch culture prior to continuous operation, $T$ is the time period during which steady-state conditions prevail, and $\bar{x}$ is the steady-state cell concentration.

Maximum output of biomass per unit time (*i.e.* productivity) in a chemostat may be achieved by operating at the dilution rate giving the highest value of $D\bar{x}$, this value being referred to as $D_{max}$. Batch fermentation productivity, as described by equation (5), is an average for the total time of the fermentation. Because $dx/dt = \mu x$, the productivity of the culture increases with time and, thus, the vast majority of the biomass in a batch process is produced near the end of the log phase. In a steady-state chemostat, operating at, or near, $D_{max}$ the productivity remains constant, and maximum, for the whole fermentation. Also, a continuous process may be operated for a very long time so that the non-productive period, $t_{iii}$ in equation (6), may be insignificant. However, the non-productive time element for a batch culture is a very significant period, especially as the fermentation would have to be re-established many times during the running time of a comparable continuous process and, therefore, $t_{ii}$ would be recurrent.

The steady-state nature of a continuous process is also advantageous in that the system should be far easier to control than a comparable batch one. During a batch fermentation, heat output, acid or alkali production, and oxygen consumption will range from very low rates at the start of the fermentation to very high rates during the late logarithmic phase. Thus, the control of the environment of such a system is far more difficult than that of a continuous process where, at steady-state, production and consumption rates are constant. Furthermore, a continuous process should result in a more constant labour demand than a comparable batch one.

A frequently quoted disadvantage of continuous systems is their susceptibility to contamination by foreign organisms. The prevention of contamination is essentially a problem of fermenter design, construction, and operation and should be overcome by good engineering and microbiological practice. ICI recognized the overwhelming advantages of a continuous biomass process and overcame the problems of contam-

ination by building a secure fermenter capable of very long periods of aseptic operation, as described by Smith.[22]

The production of growth-associated by-products, such as ethanol, should also be more efficient in continuous culture. However, continuous brewing has met with only limited success and UK breweries have abandoned such systems owing to problems of flavour and lack of flexibility.[23] The production of industrial alcohol, on the other hand, should not be limited by the problems encountered by the brewing industry and continuous culture should be the method of choice for such a process. The adoption of continuous culture for the production of biosynthetic (as opposed to catabolic) microbial products has been extremely limited. Although, theoretically, it is possible to optimize a continuous system such that optimum productivity of a metabolite should be achieved, the long-term stability of such systems is precarious, owing to the problem of strain degeneration. A consideration of the kinetics of continuous culture reveals that the system is highly selective and will favour the propagation of the best-adapted organism in a culture. Best-adapted in this context refers to the affinity of the organism for the limiting substrate at the operating dilution rate. A commercial organism is usually highly mutated such that it will produce very high amounts of the desired product. Therefore, in physiological terms, such commercial organisms are extremely inefficient and a revertant strain, producing less of the desired product, may be better adapted to the cultural conditions than the superior producer and will come to dominate the culture. This phenomenon, termed by Calcott[24] as contamination from within, is the major reason for the lack of use of continuous culture for the production of microbial metabolites.

Although the fermentation industry has been reluctant to adopt continuous culture for the production of microbial metabolites, very considerable progress has been made in the development of fed-batch systems.[25,26] Fed-batch culture may be used to achieve a considerable degree of process control and to extend the productive period of a traditional batch process without the inherent disadvantages of continuous culture described previously. The major advantage of feeding a medium component to a culture, rather than incorporating it entirely in the initial batch, is that the nutrient may be maintained at a very low

[22] R. L. Smith, *Philos. Trans. R. Soc. London., Ser. B*, 1980, **290**, 341.
[23] B. H. Kirsop, in 'Topics in Enzyme and Fermentation Biotechnology', ed. A. Wiseman, Ellis Horwood, Chichester, 1982, p. 79.
[24] P. H. Calcott, 'Continuous Culture of Cells', CRC Press, Boca Raton, Fl., 1981, Vol. 1, p. 13.
[25] A. Whitaker, *Process Biochem.*, 1980, **15**(4), 10.
[26] T. Yamane and S. Shimizu, *Adv. Biochem. Eng./Biotechnol.*, 1984, **30**, 147.

concentration during the fermentation. A low (but constantly replenished) nutrient level may be advantageous in:

(i) Maintaining conditions in the culture within the aeration capacity of the fermenter.
(ii) Removing the repressive effects of medium components such as rapidly used carbon and nitrogen sources and phosphate.
(iii) Avoiding the toxic effects of a medium component.
(iv) Providing a limiting level of a required nutrient for an auxotrophic strain.

The earliest example of the commercial use of fed-batch culture is the production of bakers' yeast. It was recognized as early as 1915 that an excess of malt in the production medium would result in a high rate of biomass production and an oxygen demand which could not be met by the fermenter.[27] This resulted in the development of anaerobic conditions and the formation of ethanol at the expense of biomass. The solution to this problem was to grow the yeast initially in a weak medium and then add additional medium at a rate less than the organism could use it. It is now appreciated that a high glucose concentration represses respiratory activity, and in modern yeast production plants the feed of molasses is under strict control based on the automatic measurement of traces of ethanol in the exhaust gas of the fermenter. As soon as ethanol is detected the feed rate is reduced. Although such systems may result in low growth rates, the biomass yield is near that theoretically obtainable.[28]

The penicillin fermentation provides a very good example of the use of fed-batch culture for the production of a secondary metabolite.[29] The penicillin process is a two-stage fermentation; an initial growth phase is followed by the production phase or idiophase. During the production phase glucose is fed to the fermentation at a rate which allows a relatively high growth rate (and therefore rapid accumulation of biomass) yet maintains the oxygen demand of the culture within the aeration capacity of the equipment. If the oxygen demand of the biomass were to exceed the aeration capacity of the fermenter anaerobic conditions would result and the carbon source would be used inefficiently. During the production phase the biomass must be maintained at a relatively low growth rate

[27] G. Reed and T. W. Nagodawithana, 'Yeast Technology', 2nd Edn, Avi, Westport, 1991.
[28] A. Fiechter, in 'Advances in Biotechnology, 1, Scientific and Engineering Principles', ed. M. Moo-Young, C. W. Robinson and C. Vezina, Pergamon Press, Toronto, 1981, p. 261.
[29] J. M. Hersbach, C. P. Van der Beek and P. W. M. Van Vijek, in 'Biotechnology of Industrial Antibiotics', ed. E. J. Vandamme, Marcel Dekker, New York, 1984, p. 387.

and, thus, the glucose is fed at a low dilution rate. Phenylacetic acid is a precursor of the penicillin molecule but it is also toxic to the producer organism above a threshold concentration. Thus, the precursor is also fed into the fermentation continuously, thereby maintaining its concentration below the inhibitory level.

## 5 THE GENETIC IMPROVEMENT OF PRODUCT FORMATION

Owing to their inherent control systems, microorganisms usually produce commercially important metabolites in very low concentrations and, although the yield may be increased by optimizing the cultural conditions, productivity is controlled ultimately by the organism's genome. Thus, to improve the potential productivity, the organism's genome must be modified and this may be achieved in two ways: by (i) classical strain improvement by mutation and selection and (ii) the use of recombination.

### 5.1 Mutation

Each time a microbial cell divides there is a small probability of an inheritable change occurring. A strain exhibiting such a changed characteristic is termed a mutant and the process giving rise to it, a mutation. The probability of a mutation occurring may be increased by exposing the culture to a mutagenic agent such as UV light, ionizing radiation, and various chemicals, for example nitrosoguanidine, nitrous acid and caffeine. Such an exposure usually involves subjecting the population to a mutagen dose which results in the death of the vast majority of the cells. The survivors of the mutagen exposure may then contain some mutants, the vast majority of which will produce lower levels of the desired product. However, a very small proportion of the survivors may be improved producers. Thus, it is the task of the industrial geneticist to separate the desirable mutants (the superior producers) from the very many inferior types. This approach is easier for strains producing primary metabolites than it is for those producing secondary metabolites, as may be seen from the following examples.

The synthesis of a primary microbial metabolite (such as an amino acid) is controlled such that it is only produced at a level required by the organism. The control mechanisms involved are the inhibition of enzyme activity and the repression of enzyme synthesis by the end product when it is present in the cell at a sufficient concentration. Thus, these mechanisms are referred to as feedback control. It is obvious that a

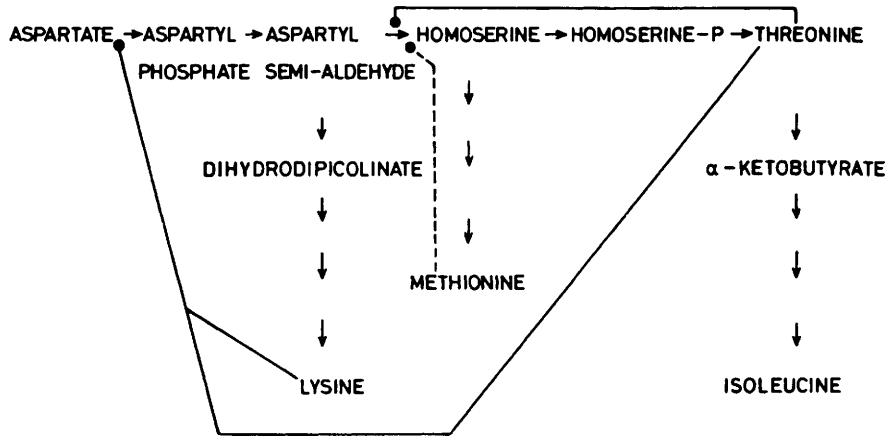


**Figure 4** *The control of biosynthesis of lysine in* Corynebacterium glutamicum: *Biosynthetic route →; Feedback inhibition —●; Feedback repression – – –●*

good 'commercial' mutant should lack the control systems so that 'overproduction' of the end product will result. The isolation of mutants of *Corynebacterium glutamicum* capable of producing lysine will be used to illustrate the approaches which have been adopted to remove the control systems.

The control of lysine synthesis in *C. glutamicum* is illustrated in Figure 4 from which it may be seen that the first enzyme in the pathway, aspartokinase, is inhibited only when both lysine and threonine are synthesized above a threshold level. This type of control is referred to as concerted feedback control. A mutant which could not catalyse the conversion of aspartic semialdehyde into homoserine would be capable of growth only in a homoserine-supplemented medium and the organism would be described as a homoserine auxotroph. If such an organism were grown in the presence of very low concentrations of homoserine the endogenous level of threonine would not reach the inhibitory level for aspartokinase control and, thus, aspartate would be converted into lysine which would accumulate in the medium. Thus, a knowledge of the control of the biosynthetic pathway allows a 'blueprint' of the desirable mutant to be constructed and makes easier the task of designing the procedure to isolate the desired type from the other survivors of a mutation treatment.

The isolation of bacterial auxotrophs may be achieved using the penicillin enrichment technique developed by Davis.[30] Under normal culture conditions an auxotroph is at a disadvantage compared with the

[30] B. D. Davis, *Proc. Natl. Acad. Sci. USA*, 1949, **35**, 1.

parental (wild-type) cells. However, penicillin only kills growing cells and, therefore, if the survivors of a mutation treatment were cultured in a medium containing penicillin and lacking the growth requirement of the desired mutant only those cells unable to grow would survive, *i.e.* the desired auxotrophs. If the cells were removed from the penicillin broth, washed, and resuspended in a medium containing the requirement of the desired auxotroph then the resulting culture should be rich in the required type. Nakayama *et al.*[31] used this technique to isolate a homoserine auxotroph of *C. glutamicum* which produced 44 g $l^{-1}$ lysine.

An alternative approach to the isolation of mutants which do not produce controlling end products (*i.e.* auxotrophs) is to isolate mutants which do not recognize the presence of controlling compounds. Such mutants may be isolated from the survivors of a mutation treatment by exploiting their capacity to grow in the presence of certain compounds which are inhibitory to the parental types. An analogue is a compound which is similar in structure to another compound and analogues of primary metabolites are frequently inhibitory to microbial cells. The toxicity of the analogue may be due to any of a number of possible mechanisms; for example, the analogue may be incorporated into a macromolecule in place of the natural product, resulting in the production of a defective compound, or the analogue may act as a competitive inhibitor of an enzyme for which the natural product is a substrate. Also, the analogue may mimic the control characteristics of the natural product and inhibit product formation despite the fact that the natural product concentration is inadequate to support growth. A mutant which is capable of growing in the presence of an analogue inhibitory to the parent may owe its resistance to any of a number of mechanisms. However, if the toxicity were due to the analogue mimicking the control characteristics of the normal end product, then the resistance may be due to the control system being unable to recognize the analogue as a control factor. Such analogue-resistant mutants may also not recognize the natural product and may, therefore, overproduce it. Thus, there is a reasonable probability that mutants resistant to the inhibitory effects of an analogue may overproduce the compound to which the analogue is analogous. Sano and Shiio[32] made use of this approach in attempting to isolate lysine-producing mutants of *Brevibacterium flavum*. The control of lysine formation in *B. flavum* is the same as that illustrated in Figure 4 for *C. glutamicum*. Sano and Shiio demonstrated that the lysine analogue

[31] K. Nakayama, S. Kituda and S. Kinoshita, *J. Gen. Appl. Microbiol.*, 1961, **7**, 41
[32] K. Sano and I. Shiio, *J. Gen. Appl. Microbiol.*, 1970, **16**, 373.

*S*-(2-aminoethyl)cysteine (AEC) only inhibited growth completely in the presence of threonine, which suggests that AEC combined with threonine in the concerted inhibition of aspartokinase and deprived the organism of lysine and methionine. Mutants were isolated by plating the survivors of a mutation treatment onto agar plates containing both AEC and threonine. A relatively high proportion of the resulting colonies were lysine overproducers, the best of which produce more than $30\ g\ l^{-1}$. A fuller account of the isolation of amino acid and nucleotide producing strains may be found in reference 3.

Thus, a knowledge of the control systems may assist in the design of procedures for the isolation of mutants overproducing primary metabolites. The design of procedures for the isolation of mutants overproducing secondary metabolites has been more difficult owing to the fact that far less information was available on the control of production and, also, that the end products of secondary metabolism are not required for growth. Thus, many current industrial strains have been selected using direct, empirical, screens of the survivors of a mutation treatment for productivity rather than cultural systems which give an advantage to potential superior producers. A programme typical of the industry in the 1950s to 1970s is illustrated in Figure 5. The throughput of such programmes has been increased by miniaturizing[3] the systems. Small volumes of liquid media in tubes or microtitre plates have been used coupled with the use of robots to automate the process. However, as more information has accumulated on the biosynthesis and control of secondary metabolites, directed selection approaches have also been used, thus reducing the empirical nature of the screens. Mutants capable of producing increased levels of secondary metabolite precursors have been isolated by techniques similar to those used for the improvement of primary metabolite producers. For example, analogue-resistant mutants have been isolated giving improved yields of pyrrolnitrin, candicidin and cephamycin.[3] Relief of carbon repression has been achieved on of mutants resistant to 2-deoxyglucose, a glucose analogue.[33] Further examples of selection methods for the isolation of improved secondary metabolite-producing strains are given in reference 3.

## 5.2 Recombination

Hopwood[34] defined recombination as any process which helps to generate new combinations of genes that were originally present in

[33] D. A. Hodgson, *J. Gen. Microbiol.*, 1982, **128**, 2417.
[34] D. A. Hopwood in 'Genetics of Industrial Micro-organisms', ed. O. K. Sebec and A. J. Laskin, American Society of Microbiology, Washington, DC, 1979, p. 1.

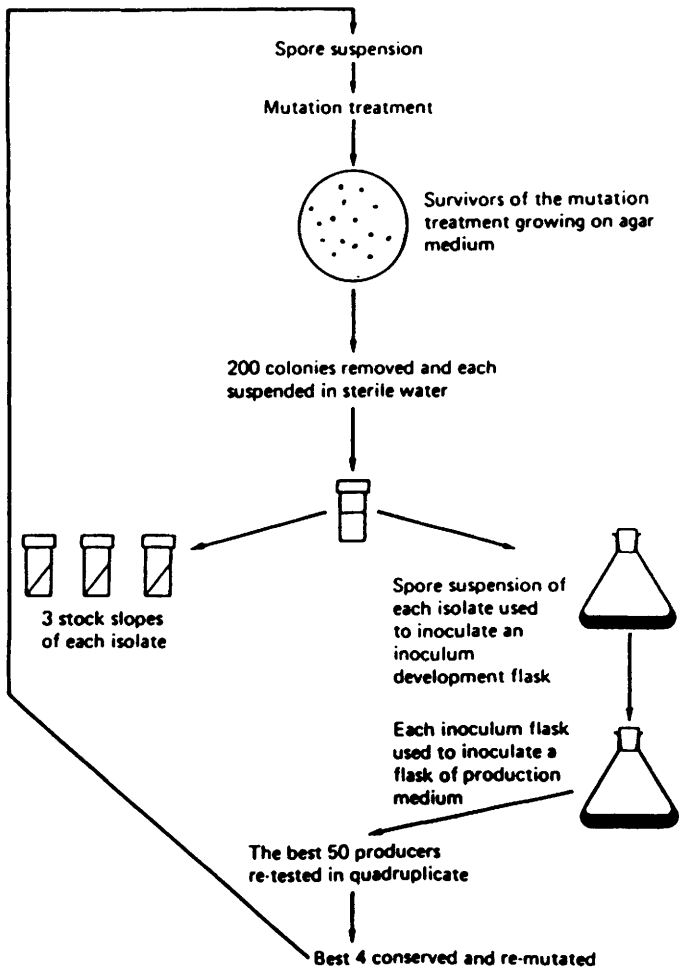


**Figure 5** *A strain improvement programme for a secondary metabolite producing culture* (Reproduced with permission from P. F. Stanbury, A. Whitaker and S. J. Hall, 'Principles of Fermentation Technology', 2nd Edn, Pergamon Press, Oxford, 1995)

different individuals. Compared with the use of mutation techniques for the improvement of industrial strains the use of recombination was fairly limited in the early years of improvement programmes. However, techniques are now widely available which allow the use of recombination as a system of strain improvement. *In vivo* recombination may be achieved in the asexual fungi (*e.g. Penicillium chrysogenum*, used for the commercial production of penicillin) using the parasexual cycle.[35] The

[35] K. D. Macdonald and G. Holt, *Sci. Prog.*, 1976, **63**, 547.

technique of protoplast fusion has increased greatly the prospects of combining together characteristics found in different production strains. Protoplasts are cells devoid of their cell walls and may be prepared by subjecting cells to the action of wall-degrading enzymes in isotonic solutions. Cell fusion, followed by nuclear fusion, may occur between protoplasts of strains which would not otherwise fuse and the resulting fused protoplast may regenerate a cell wall and grow as a normal cell. Protoplast fusion has been achieved using filamentous fungi, yeasts, streptomycetes and bacteria. For example, Tosaka *et al.*[36] improved the rate of glucose consumption (and therefore lysine production) of a high lysine producing strain of *B. flavum* by fusing it with another *B. flavum* strain which was a non-lysine producer but consumed glucose at a high rate. Among the fusants one strain exhibited high lysine production with rapid glucose utilization. Chang *et al.*[37] used protoplast fusion to combine the desirable properties of two strains of *P. chrysogenum* producing penicillin V into one producer strain. Lein[38] has described the use of protoplast fusion for the improvement of penicillin production in the procedures used by Panlabs Inc. and DeWitt *et al.*[39] reviewed the technique for the improvement of actinomycete processes.

*In vitro* recombination has been achieved by the techniques of *in vitro* recombinant DNA technology discussed elsewhere in this book. Although the most well publicised recombinants achieved by these techniques are those organisms which synthesize foreign products (see Section 3.5), very considerable achievements have been made in the improvement of strains producing conventional products. The efficiency of *Methylomonas methylotrophus*, the organism used in the ICI Pruteen process, was improved by the incorporation of a plasmid containing a glutamate dehydrogenase gene from *E. coli*.[40] The manipulated organism was capable of more efficient ammonia metabolism which resulted in a 5% improvement in carbon conversion. However, the strain was not used on the large-scale plant due to problems of scale-up.

*In vitro* DNA technology has been used to amplify the number of copies in a critical pathway gene (or operon) in a process organism. Although gene amplification is not an example of recombination it is best

[36] O. Tosaka, M. Karasawa, S. Ikeda and Y. Yoshii, Abstracts of 4th International Symposium on Genetics of Industrial Microorganisms, 1982, p. 61.
[37] L. T. Chang, D. T. Terasaka and R. P. Elander, *Dev. Ind. Microbiol.*, 1982, **23**, 21.
[38] J. Lein, in 'Overproduction of Microbial Metabolites', ed. Z. Vanek and Z. Hostalek, Butterworths, Boston, 1986, p. 105.
[39] J. P. DeWitt, J. V. Jackson and T. J. Paulus, in 'Fermentation Process Development of Industrial Organisms', ed. J. O. Neway, Marcel Dekker, New York, 1989, Chapter 1, p. 1.
[40] J. D. Windon, M. J. Worsey, E. M. Pioli, D. Pioli, P. T. Barth, K. T. Atherton, E. C. Dart, D. Byrom, K. Powell and P. J. Senior, *Nature (London)*, 1980, **287**, 396.

considered in the context of DNA manipulative techniques. Threonine production by *E. coli* has been improved by incorporating the entire threonine operon of a threonine analogue-resistant mutant into a plasmid which was then introduced back into the bacterium. The plasmid copy number in the cell was approximately 20 and the activity of the threonine operon enzymes was increased 40 to 50 times. The organism produced 30 g $l^{-1}$ threonine compared with the 2–3 g $l^{-1}$ of the non-manipulated strain.[41] Miwa *et al.*[42] utilized similar techniques in constructing an *E. coli* strain capable of synthesizing 65 g $l^{-1}$ threonine.

The application of the techniques of genetic manipulation to the improvement of *C. glutamicum* (see Section 5.1) was hindered by the availability of a suitable vector. However, vectors have been constructed and considerable progress has been made in the improvement of amino acid fermentations.[3] Threonine, histidine and phenylalanine production have been improved using gene amplification techniques. In these examples the cloned genes were mutant forms which were resistant to feedback control and had been obtained using the conventional mutagenesis/screening systems described in Section 5.1. Thus, the *in vitro* DNA techniques have built upon the achievements of conventional strain improvement.

Phenylalanine has become a very important fermentation product because it is a precursor in the manufacture of the sweetener, aspartame. Backman *et al.*[43] described the rationale used in the construction of an *E. coli* strain capable of synthesizing commercial levels of phenylalanine. *Escherichia coli* was chosen as the producer because of its rapid growth, the availability of DNA manipulative techniques, and the extensive genetic database. Several of the phenylalanine genes are subject to control by the repressor protein of the *tyr* R gene. *In vitro* techniques were used to generate *tyr* R mutations and introduce them into the production strain. The promoter of the *phe* A gene, was replaced to remove repression and attenuation control. As an alternative to the traditional technique of generating a tyrosine auxotroph an excision vector carrying the *tyr* A gene was incorporated into the chromosome. The vector is excised from the chromosome at a slightly increased temperature. Thus, auxotrophy may be induced *during* the fermentation by careful temperature manipulation, thus allowing tyrosine limitation

[41] V. G. Debabof, in 'Overproduction of Microbial Products', ed. V. Krumphanzl, B. Sikyta and Z. Vanek, Academic Press, London, 1982, p. 345.

[42] K. Miwa, S. Nakamori and H. Momose, Abstracts of 13th International Congress of Microbiology, Boston, USA, 1982, p. 96.

[43] K. Backman, M. J. O'Connor, A. Maruya, E. Rudd, D. McKay, R. Balakrishnan, M. Radjai, V. Di Pasquantonio, D. Shoda, R. Hatch and K. Venkatasubramanian, *Ann. N. Y. Acad. Sci.*, 1990, **589**, 16.

to be imposed after the growth phase and at the beginning of the production phase. However, the final step in the genetic manipulation of the organism was the traditional step of isolating an analogue-resistant mutant to relieve the feedback inhibition of DAHP synthase by phenylalanine.

The application of *in vitro* recombinant DNA technology to the improvement of secondary metabolite formation is not as developed as it is in the primary metabolite field. However, considerable advances have been made in the genetic manipulation of the streptomycetes[44] and the filamentous fungi[45] and a number of different strategies have been devised for cloning secondary metabolite genes.[46] The first such genes which were cloned were those coding for resistance of the producer organism to its own antibiotic.[47] Complete antibiotic synthesizing pathways have now been cloned and the genes for antibiotic biosynthesis have been shown to be clustered together on the chromosome in both prokaryotes and eukaryotes.[48] Baltz[49] cited the application of recombinant DNA technology to the development of improved strains for the production of tylosin, pristinamycin and daptomycin and the discovery of several global and pathway specific secondary metabolism regulatory genes has opened the way to a new means of increasing yields.

## 6 CONCLUSIONS

Thus, microorganisms are capable of producing a wide range of products, a range which has been increased by the techniques of *in vitro* recombinant DNA technology to include mammalian products. Improved productivity may be achieved by the optimization of cultural conditions and the genetic modification of the producer cells. However, a successful commercial process for the production of a microbial metabolite depends as much upon chemical engineering expertise as it does on that of microbiology and genetics.

[44] K. F. Chater, *Microbiology*, 1998, **144**, 727.
[45] C. A. M. J. J. van den Hondel and P. J. Punt, in 'Applied Molecular Genetics of Fungi', ed. J. F. Peberdy, C. E. Caten, J. E. Ogden and J. W. Bennett, Cambridge University Press, Cambridge, 1991, p. 1.
[46] I. S. Hunter and S. Baumberg, in 'Microbial Products: New Approaches', ed. S. Baumberg, I. S. Hunter and P. M. Rhodes. Society for General Microbiology Symposium, Cambridge University Press, Cambridge, 1989.
[47] I. S. Hunter in 'Fermentation Microbiology and Biotechnology' ed. E. M. T. El-Mansi and C. F. A. Bryce, Taylor and Francis, London, 1999, p. 121.
[48] J. Thompson, T. Kieser, J. M. Ward and D. A. Hopwood, *Gene*, 1982, **20**, 51.
[49] R. H. Baltz, *Trends in Microbiology*, 1998, **6**(2), 76.