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The Effect of Aeration upon the Secondary Metabolism of Microorganisms

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Introduction

The authors' interest in this topic stemmed from earlier observations in these laboratories that the metabolic behaviour of fungi grown in liquid culture was very different, depending on whether they grew, without shaking, as mycelial mats or whether they grew as pellets in shake cultures (Woodhead and Walker, 1975). Culture conditions affected both the yield of secondary metabolites and extracellular enzymes. This review examines more recent work on these topics.

Oxygen and mycelial growth

Oxygen makes up 28% of the earth's atmosphere by volume and is vital for the survival of aerobic organisms due to its role in respiration but, because the respiratory enzymes of microbial cells are in an aqueous environment, cells are only able to utilize dissolved O_2 (DO). At 20°C and in an atmosphere of air, water holds only 9 ppm O_2, this amount decreasing with increasing temperature, but essentially independent of the presence and pressure of other gases (Finn, 1954).

Cell respiration is independent of the DO concentration, provided that this exceeds a threshold level (ie the critical oxygen tension) below which the rate of oxygen uptake follows Michaelis-Menten kinetics which correlates to the high O_2 affinity of

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Abbreviations: 6-MSA, 6-methylsalicylic acid; 6PGD, 6-phosphogluconate dehydrogenase; ADP, adenosine di-phosphate; ALDH, alcohol dehydrogenase; AMP, adenosine monophosphate; ATP, adenosine triphosphate; cAMP, cyclic AMP; DAOCS, deacetoxy-cephalosporin C synthase; DMAP, dimethylallyl phosphate; DO, dissolved O_2; EC, energy charge; $FADH_2$, flavin adenosine dinucleotide; G6PDH, glucose-6-phosphate dehydrogenase; HMG CoA, hydroxy-methyl-glutaryl CoA; ICDH, iso-citrate dehydrogenase; IPP, isopentenyl phosphate; k_La, volumetric coefficient; LiP, lignin peroxidase; MnP, Mn-peroxidase; NAD^+, nicotinamide adenine dinucleotide; $NADP^+$, nicotinamide adenine dinucleotide phosphate; NMR, nuclear magnetic resonance spectroscopy; OAR, O_2 absorption rate; PEP, phosphoenol pyruvate; PFK, phosphofructokinase; PPS, pentose phosphate shunt; RNA, ribose nucleic acid; SDH, succinate dehydrogenase; UAS, upstream activation sites.

Biotechnology and Genetic Engineering Reviews – Vol. 17, August 2000
0264–8725/00/17/281–323 $20.00 + $0.00 © Intercept Ltd, P.O. Box 716, Andover, Hampshire SP10 1YG, U.K.

the terminal oxidase of the respiratory electron transport chain (Harrison, 1972). For unicellular organisms, this critical DO level is low, and the respiration rate is limited more by the non-saturation of enzyme active sites than the slow diffusion of O_2 into the protoplasm. The respiration rate of multicellular organisms is more likely to be affected by the rate of diffusion of oxygen, and the supply of this substrate will dictate the rate of respiration. Thus, the demand for oxygen by a submerged microbial culture is dependent on a number of factors, including the concentration and form of nutrients in the medium, the accumulation of toxic end products, the loss of volatile intermediates, and the supply of O_2 to the medium (Finn, 1954).

The ionic strength of the culture media has been shown to affect the transfer and behaviour of O_2, with increasing ionic strength resulting in decreased O_2 solubility (Hansberg and Aguirre, 1990). Robinson and Wilke (1973) showed that variations in the ionic strength affected properties such as viscosity, density and diffusion in a basal salt medium, and were able to predict the volumetric coefficient (k_La) for mass transfer from dispersed gas bubbles to the fermentation medium. The volumetric mass transfer coefficient is the main parameter describing the performance of a bioreactor, since liquid-phase resistance controls the overall O_2 transfer rate (Kawase and Moo-Young, 1988). The dispersion of air bubbles in aqueous electrolyte solutions has been studied by Zieminski and Whittemore (1971), who showed that the degree of coalescence of bubbles in the media depended on the concentration of salts, so that the rate of O_2 transfer increased with increasing salt concentration. Anionic surface-active agents were shown to have an adverse effect on the rate of solution of O_2 in aerated water (Downing *et al.*, 1957).

Several resistances are encountered as O_2 is transferred from the gas phase to the cellular site of utilization. The main resistance is due to stagnant films, μm to mm in thickness, which are dependent on the degree of turbulence and the physical properties of the medium, and these occur between the bulk of the gas and the gas-liquid interface, and between the gas-liquid interface and the bulk of the liquid. There are liquid films around individual cells or cell clumps, as well as intracellular and intra-clump resistances (Finn, 1954). Resistances are also present in the form of the area of gas-liquid contact, the time of contact, and the intensity of agitation of the culture medium.

Whether a cell grows singly, or in a clump (as a mycelial aggregate), can be a factor in the resistance encountered in the surrounding liquid films. For cell clumps, liquid films are more important, since there is more likely to be relative motion between a clump and its surrounding fluid, and the intra-clump resistance is considerably greater than that of the liquid film. Anaerobic conditions may develop within a clump once a certain size is reached, and the O_2 uptake into a clump will determine its size. However, it must also be noted that shear forces can contribute to the breakdown of cell clumps.

Olsvik *et al.* (1993) studied clumps in *Aspergillus niger* fermentations by image analysis and showed that 89–99% of the fungal material was growing in clumps, although visually, the growth form appeared to be fully filamentous (this level of clumped growth was predicted to be common in mycelial liquid cultures of filamentous fungi). At constant biomass concentrations, the consistency and size of clumps appeared to be related to its roughness and compactness. Roughness correlated to specific growth rate of the culture, and increased with increasing O_2 tension. The O_2

was possibly having an effect on hyphal-hyphal interactions within the clumps or on the rigidity of cell walls, and not involved in clump breakdown. It was suggested by Zetelaki and Vas (1968) that the DO tension may affect cell wall composition, and therefore the flexibility of the hyphae. Oxygenated cultures of *A. niger* had less well-defined and less rigid cell walls than aerated cultures, therefore making them more resistant to mechanical agitation, resulting in lower viscosity.

Several authors have been concerned with the effect microbes have on the O_2 k_LA. Wise *et al.* (1969) showed that, in medium aerated at a low Reynolds number flow (ie laminar flow-type conditions; the Reynolds number is a dimensionless indicator of turbulence within the system, and is a means for describing the flow regime in the system). According to Banks (1977), the rate of O_2 transfer was increased by the presence of microbial cells at the liquid surface film. Because the physical properties of the medium were not changed by the fermentation, it was proposed that this effect was due to the disruption of the liquid surface film around the O_2 bubbles. The organisms used were *Candida intermedia* and *Pseudomonas ovalis*, and similar results have been reported with unicellular organisms such as *Escherichia coli* (Andrews *et al.*, 1984). King and Palmer (1989) concluded that, because increased O_2 transfer was observed with *Bacillus licheniformis*, but not *Micrococcus luteus*, the accumulation effect was related to motility. Ju and Sundararajan (1995) showed the overall effect of *Saccharomyces cerevisiae* cells on O_2 uptake was positive, and was not due to their presence in the layer near the bubble surface (which was considered to reduce permeability to O_2), but instead was due to cell respiration and medium modification, contrary to the findings of Wise *et al.* (1969). Furthermore, since the observed effects occurred at high agitation speeds and low aeration rates, they were attributed to the interfacial cell accumulation associated with the smaller bubbles produced under these conditions. The blocking effect of non-respiring cells was also observed in previous work by these authors (Ju and Sundararajan, 1991), and also by Huang and Bungay (1973), who showed the presence of a stagnant 'skin' which gave resistance to O_2 transfer in static cultures of *P. ovalis*.

The nature of O_2 uptake has also been studied in yeast cultures in which an increased rate of uptake had been observed. Sobotka *et al.* (1981) proposed a two-phase uptake model for aerobic fermentations whereby O_2 uptake can occur from both the liquid and gaseous phases. A model was derived for two-phase uptake and provided a better fit for the observed results than if a liquid phase only was considered.

During the course of penicillin-producing fermentations by *Streptomyces griseus*, the O_2 uptake rate changed throughout the life cycle of the organism and the accompanying changes in fermentation conditions (Bartholomew *et al.*, 1950). Ju *et al.* (1991) showed that changes in CO_2 composition in the gas supply to *Penicillium chrysogenum* fermentations had a dramatic effect on both broth rheology and k_La, since this fungus varied in form from normal mycelia to pellets; this might be useful in controlling culture morphology and, therefore, O_2 transfer capabilities.

AGITATION AND AERATION

Improvements in the interfacial exchange between gas and liquid can be brought about by agitation. The air stream is broken into smaller bubbles, thus increasing the area available for O_2 transfer, and swift eddies in the circulating media prevents the

escape and coalescence of bubbles. The turbulent shear created also reduces the thickness of the liquid film (Banks, 1977). Further reduction of resistance can be gained by increased partial pressure of O_2 (pO_2) using O_2-enriched air or a higher air pressure, but the latter has the undesirable consequence of the sweeping effect of nitrogen, and increased solubility of CO_2. Inadequate CO_2 can become a problem when a pure O_2 atmosphere is used (Finn, 1954).

In liquid culture, unicellular microorganisms, such as yeast and bacteria, are considered to behave as Newtonian fluids, which means they follow Newton's Law of Viscous Flow so that the velocity gradient in the fluids is directly proportional to the shear stress. When a force is applied to a liquid, it will flow and the viscosity of the liquid may be considered as its inherent resistance to flow (Banks, 1977). Single cells have a liquid film at the cell wall, which offers no resistance to the diffusion of O_2. The thickness of this film can be reduced only by increasing the relative velocity between the cell and the fluid. However, the cells follow fluid streamlines since their specific activity in the culture medium is not sufficient enough to overcome the inertia due to their small size. In this case, increased growth rate is due to agitation side effects, such as relief of CO_2-saturation of the culture medium caused by the organism (Finn, 1954).

For fungi, the effect is completely different. Fungal mycelia are considerably larger than unicells and greater in size than the eddies in which the turbulent energy in fermentors is dissipated, thus dampening the turbulence and, consequently, the k_La (Andrews *et al.*, 1984). Mycelial cultures behave as non-Newtonian fluids and do not possess a 'true' viscosity, only an 'apparent' viscosity, which is not a constant, characteristic property of the fluid and which is dependent on the shear rate. Banks (1977) described four non-Newtonian fluids of interest to fermentation technologists:-

(1) Bingham plastics: similar to a Newtonian fluid but different because there is a limiting shear stress, which must be exceeded if a liquid flow is to occur. At rest, these fluids have a three-dimensional structure of sufficient rigidity to withstand any stress less than the yield stress. However, once the yield stress is exceeded the fluid behaves in a Newtonian manner.
(2) Pseudoplastics: the apparent viscosity decreases with shear rate, an example of which is polymer solutions (which could be analogous to hyphal solutions) where, at progressively higher shear rates, the long chain molecules of the polymer tend to align with each other and thus slip more readily, causing decreased viscosity. Cultures of *Streptomyces niveus* were O_2-limited despite a 70% DO saturation since the viscosity of a pseudoplastic liquid increases with increasing shear stress, resulting in broth viscosity increasing with distance from the impeller in a fermentor (Bushell, 1989).
(3) Dilatants: the apparent viscosity increases with increasing shear rate.
(4) Casson body: these possess a well-defined yield stress whilst the apparent viscosity decreases with increasing shear rate, ie combining the properties of a Bingham plastic and a pseudoplastic. This occurs in many concentrated suspensions.

Mycelial suspensions exhibit non-Newtonian properties because the branched mycelium forms a three-dimensional structure that gives the suspension rigidity,

resulting in a yield stress. The long hyphae tend to align with each other with increasing shear rate, appearing pseudoplastic in nature. Most published work indicates that cultures of hyphal organisms behave either as pseudoplastics, Bingham plastics or Casson bodies (Banks, 1977). The behaviour of the culture can change according to its age or stage of the fermentation, and a *Streptomyces griseus* culture fluid behaved as either a Newtonian or a Bingham plastic depending on age; Bingham plastic occurred approximately midway through the fermentation, and Newtonian behaviour was displayed during the initial and final stages of the fermentation. The final period of the culture was Newtonian due to increased lysis and fragmentation of the mycelium (Banks, 1977). During the course of any fermentation, the cell density will increase and the morphological characteristics of the organism, such as degree of mycelial branching, will alter and affect the culture rheology. In the initial stages of the fermentation, the organism will be distributed throughout the medium as small, discrete, mycelial agglomerates, in which case the culture fluid will behave as a Newtonian fluid. Later, once the three-dimensional hyphal structure has formed, non-Newtonian behaviour will result and finally, as mentioned earlier, partial or complete lysis will occur and the culture would be expected to revert to Newtonian behaviour (Banks, 1977).

According to Banks (1977), there are a number of difficulties with cultures that exhibit non-Newtonian fluid behaviour. The first is that the high viscosity of many fungal and streptomycete cultures prevents fully developed turbulent flow from being established, so that within such cultures the flow conditions in the fermentor lie in a transition region between viscous and turbulent flow. In the transition region, the relationship between operating variables and power consumption are complex, and little is known about the relationship between power consumption and aeration efficiency. Secondly, non-Newtonian fluids do not possess a true viscosity, only an apparent viscosity which is not constant and is dependent upon shear rate. Since shear rates vary within the fermentor, the apparent viscosity will also be variable and, therefore, a complex system will exist which defies theoretical analysis. Thirdly, there is a lack of information available concerning the rheological behaviour of filamentous microorganisms.

Under normal operating conditions, the O_2 efficiency of a culture seldom exceeds 2%, and is often below 1%. On a small scale, shake flasks are adequate because O_2 transfer takes place by a diffusion process through the surface liquid film which is constantly renewed under such conditions (Banks, 1977). Shaking is also considered better than bubbling devices since there is a shallow layer of culture medium. However, a major disadvantage of this method is the production of head foam (Finn, 1954). Shaking is impractical on a large scale because the liquid surface area/volume ratio decreases with increasing scale, so the best form of aeration in this situation is provided by stirred fermentors (Banks, 1977).

Two forms of shaking devices have been utilized for the aeration of submerged cultures; the reciprocating shaker and the rotary shaker. The former is less favoured than the latter, a major problem being inconsistent fluid surges in which a wave-like curl return causes unpredictable and uncontrollable 'geyser'-like splashes. There are often substantial viscosity effects, and evaporation of the medium frequently affects the motion of the fluid being shaken. The rotary shaker creates a constant swirl pattern, which is less affected by media viscosity, machine start-up,

or flask volume than a reciprocating system. It is also better for heavy and non-uniform loads.

Freedman (1970) determined the extent of sodium sulphite oxidation in shake flasks to calculate O_2 absorption rates (OARs). This work showed that the OAR increased with increased shaker speeds (best above 300 rpm), increased temperature, and when lower flask volumes were used (media volume 10% of flask volume). Smaller flasks (10 and 25 ml) gave the best OAR results and, interestingly, the commonly used 250 ml Erlenmeyer flasks gave the poorest results! The size of the stroke of the rotation was also important; the larger the stroke, the better the OAR. Baffled flasks gave better OAR values, but resulted in less uniform cultures.

A system using sparged aeration in shaken flasks was described by Donovan *et al.* (1995); this retained the advantages of the shake flask method but the overall performance was improved by aeration of the broth during shaking. The authors claimed a doubling of growth rate of *E. coli* cells by 8 hours in the sparged system over the non-sparged cultures.

A different means of increasing aeration efficiency was demonstrated by Gbewonyo and Wang (1983) who studied penicillin production by *P. chrysogenum* grown in a 3 litre bubble column fermentor. The fungus was allowed to grow, either as free cells, or confined to Celite® beads, and the results showed that the restructuring of the filamentous morphology of the cells resulted in significantly higher k_La values, growth rate and penicillin production than for the free cell cultures. The specific energy consumption of the process was almost halved in the Celite® bead cultures and required less input aeration. These results showed that free mycelial growth caused a decline in the mass transfer capacity attributed to the high non-Newtonian viscosities of the fermentation medium that resulted.

Over-agitation, or over-aeration, may occur. The altered yields attributed to this have been difficult to interpret since other factors such as evaporative cooling of the culture, pH changes, foaming and/or the use of anti-foaming agents, acceleration of the degradative reactions and the loss of volatile intermediates such as acetaldehyde or CO_2, may all have an effect (Finn, 1954). Mycelial age is important where over-agitation is concerned since older, more fragile, hyphae may be prone to greater lysis under high speeds, whereas younger mycelia tend to be more robust and unlikely to rupture (unless abrasive particles are present).

OXYGEN AND PELLETING

Under certain conditions, submerged fungal cultures grow in the form of spherical, compact masses of hyphal pellets. Pelleting has been attributed to low concentrations of conidial inocula (Camici *et al.*, 1952), for example $2–3 \times 10^5$ conidia/ml for *P. chrysogenum* (Trinci, 1970). It is thought to be dependent on the fungal strain, but nutrient media and low agitation have also been shown to have an effect (Phillips, 1966). Olsvik *et al.* (1993) observed small pellets (less than 5 mm) of *A. niger* occurring at 40% DO in the broth, and at a low growth/dilution rate in a fungus which otherwise tended to grow in clumps.

Many fungi form pellets when grown in shaken submerged cultures, probably due to an aggregation process which may occur between small clumps and through entanglement of spores in hyphae (Trinci, 1970). Nielsen (1996) ranks three groups

of pellet-forming microorganisms according to their mechanism of pellet development:

(1) Spore coagulating type, where germination of coagulated spores results in a pellet being formed (eg many *Aspergillus* species);
(2) The non-coagulating type, where a single spore forms a pellet (eg some *Streptomyces* species); and
(3) The hyphal element agglomerating type, where hyphal elements agglomerate and form a clump of hyphal elements which eventually form a pellet (eg *P. chrysogenum*).

Pelleting helps maintain low viscosity and high aeration efficiency, although the nature of pellet morphology prevents effective utilization of the available O_2. Phillips (1966) showed that with *P. chrysogenum* the larger the pellet, the lower the critical O_2 tension in the interior, but the greater the DO of the broth, and *vice versa*.

Trinci (1970) proposed zones relating to growth efficiencies within *A. nidulans* pellets, illustrated in *Figure 11.1*, and he explained these by using cube root growth kinetics for fungal pellets as originally proposed by Pirt (1966). Cube root growth kinetics is a feature of pelleted growth, which is affected by the texture/density of the pellets concerned; for example, pellets with densely packed hyphae would allow only the simple diffusion of nutrients and O_2 to the interior, whilst pellets with a looser texture could be supplied with nutrients and O_2 by the turbulence of the medium.

According to Trinci (1970), an outer growth zone grows exponentially at the maximum specific growth rate, with only the outer portion of this region actually contributing to the radial growth of the pellet. Anaerobic conditions are assumed to

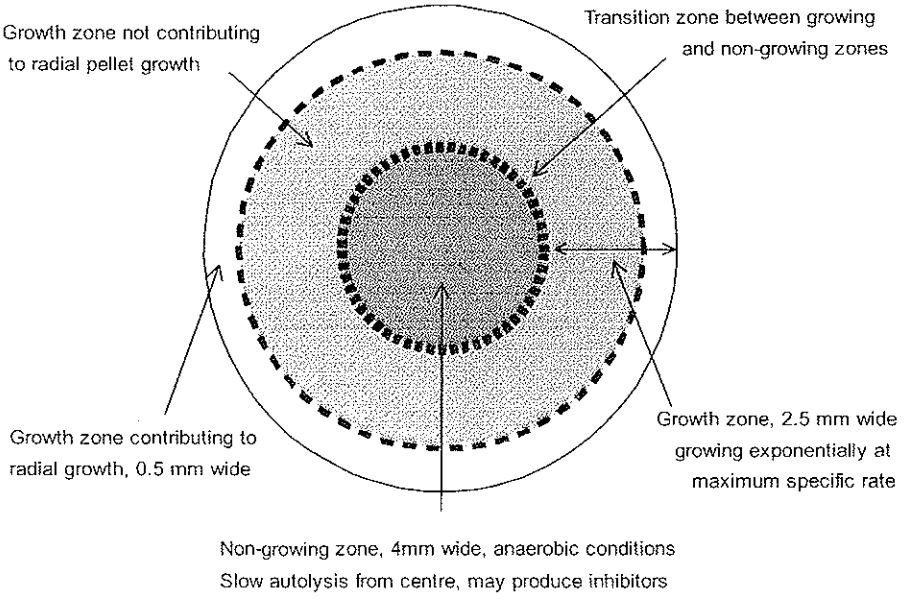

Figure 11.1. Zones of growth in fungal pellets (redrawn from Trinci, 1970).

prevail in the interior, leading to degenerative processes such as autolysis, or production of growth-inhibitory substances, the diffusion of which may contribute to the deceleration of radial growth rate at a specific diameter. Autolysis of pellet interiors leading to an inner space has been reported previously (Camici *et al.*, 1952) and, in a *P. urticae* strain, sporulation occurred at the centre of the pellets by a process analogous to the formation of arthrospores at the central region of surface cultures and where both regions were considered to be deprived of O_2 (Yanagita and Kogane, 1963).

Hansberg and Aguirre (1990) produced a hypothesis to account for differentiation processes in cells and which appears to be relevant here. This states that cells are in a stable state during growth and once they have reached a differentiated state. However, the switch from one to the other requires instability, and a hyperoxidant state is proposed as the causative mechanism for this. Hyperoxidant states come about due to the production of excess reactive O species, such as $^1O_2$, O_2^-, HO_2, H_2O_2, and HO, which are unable to be neutralized adequately by the cell, or if excess O_2 is able to enter the cell and therefore its metabolism. It is believed that a hyperoxidant state could contribute to processes such as protein carbonylization, which have been shown to coincide with different phases of development. Inherent in this theory is that, once a cell reaches a differentiated state, or is part of a differentiated structure, it becomes isolated from the environment and, therefore, any O_2 that may be present. The differentiation processes that occur inside pellets could be due to this, but their internal environments could be expected to be less aerobic so that such a process might not be relevant. In effect, the pellet interior is more or less isolated from O_2 so that the individual differentiated cells fit this theory.

A process which occurs as a result of the non-growing central region of the pellet is pellet break-up, which is attributed to the age of the hyphal pellet with respect to the amount of substrate depletion that has occurred at the centre, and to how fast cell lysis has occurred in this region. Shear forces in the growth media may also play a role in this process (Nielsen, 1996).

Erosion of the pellet surface could also occur as a result of culture hydrodynamics and this could account for the rupture of hyphae at the pellet surface, resulting in a reduction in the ability to interact with the surrounding medium (Nielsen, 1996).

Huang and Bungay (1973) used a microprobe to establish that the depth at which O_2 was limiting in *A. niger* pellets was 100 μm. However, this work was done on a mounted pellet for which the flow velocity of the medium would have been in excess of that expected for a free pellet in a standard fermentation, and therefore provided a greater O_2 supply than normal. Increased pellet sizes resulted in an increase in specific respiration rate for *A. niger* pellets and the apparent K_m for O_2 for respiration. This lead to the conclusion that mycelia in the centre of the pellets were adapted to the low O_2 concentration, therefore discounting the possibility that there might have been uniform respiration throughout the pellet, or that respiratory capacity was affected by age (Kobayashi *et al.*, 1973).

FUNCTIONS OF O_2 IN MICROORGANISMS

O_2 performs a major role in aerobic organisms as the terminal electron acceptor in the electron transport chain. It is utilized in a sequential mechanism whereby 2 electrons

(originally from the Krebs/TCA cycle as NADH + H^+) are transferred from the cytochrome a_3 of cytochrome oxidase to form O^{2-}, which associates with 2 H^+ from $FADH_2$ (produced in succinate dehydrogenase and $NADH_2$ reductase reactions) to form H_2O. Cytochromes play an important role in respiratory metabolism and their levels have been shown to adapt in response to varied O_2 tensions. Harrison (1972) reviewed several bacterial and yeast (including obligate aerobes and facultative organisms) cytochrome responses to various DO concentrations and concluded that, with the exception of one culture, a maximum cytochrome content was present in cells under O_2-limited conditions. Facultative anaerobic bacteria also contain cytochromes that can be damaged by O_2, thus indicating the importance of the critical O_2 tension (Mukhopdhyay and Ghose, 1976).

O_2 also functions as a nutrient for some microbes. Yeasts and fungi incorporate O_2 into unsaturated fatty acids and sterols, which are essential for cell growth and where anaerobic conditions lead to the inability to form these compounds, it is necessary to add unsaturated fatty acids to the medium. At low O_2 concentrations, this nutritional requirement leads to competition between synthetic and respiratory pathways. Methane-utilizing bacteria also require oxygen and obtain it from DO and O-containing compounds in the growth medium. The methane carbon atom is oxidized by oxygenase enzymes (Harrison, 1972). Another example is catechol-2,3-dioxygenase, produced by some *Pseudomonas* species, and which can function in hypoxic environments utilizing NO_3^- as an electron acceptor in the absence of O_2. The kinetic parameters of this enzyme reflect the nature of the environment, allowing it to function, and its synthesis is in response to limiting O_2 concentrations (Kukor and Olsen, 1996).

The regulation of metabolism in facultative anaerobes is another role for O_2. Medium pO_2/DO levels induce/repress pathway enzymes in these organisms, and the inductive effects occur at levels well below the critical level for respiration. Facultative anaerobes continue to follow anaerobic pathways for biosynthetic reactions; yet, whenever possible, they use O_2 as a terminal electron acceptor, thus repressing the synthesis of systems required for anaerobic energy metabolism (Mukhopdhyay and Ghose, 1976).

O_2 can also act as an energy generator in microorganisms in two ways. Firstly, biothermo-chemical reactions can occur to generate heat, which can be converted into electrical energy. Glucose and O_2 are converted to CO_2 and water, yielding 3.72 MJ $mole^{-1}$ and ATP is synthesized by oxidative phosphorylation.

Finally, O_2 can control the rate of active transport in the cell through DO concentrations at the cell surface which, in turn, controls product synthesis in oxygen-requiring pathways as a cellular mechanism (Mukhopdhyay and Ghose, 1976).

MECHANISMS OF RESPONSE TO DISSOLVED O_2 LEVELS

Harrison (1972, 1976) has reviewed the mechanisms by which cells adjust to different DO levels and a summary of his conclusions is presented here. Four levels of response over time can be distinguished in growing microbes.

Within seconds, a primary response with no feedback control occurs where molecules in the organism undergo chemical or physico-chemical interactions with the change in the environment; this follows Michaelis-Menten kinetics and few changes occur this way.

After less than 15 minutes, feedback control of metabolic pathways can occur and this is often dictated by changes in adenine nucleotide levels. The ATP/ADP ratio was established as an expression of energy status to indicate overall metabolic direction, but it is not an accurate expression of available energy phosphate bonds since adenylate kinase catalyzes a freely reversible reaction between 2 ADP and ATP + AMP, which acts to buffer the levels of these three molecules. Subsequently, Atkinson's hypothesis was formulated to describe the effects of changes in adenine nucleotide levels on energy charge (EC) to describe the cell's metabolic state (Chapman *et al.*, 1971). Energy charge is defined as EC = [ATP + ½ ADP]/[ATP + ADP + AMP] (Atkinson and Walton, 1967). Decreases in EC favour catabolic processes generating more high-energy bonds as ATP, whilst rises in EC favour anabolic processes which utilize the high-energy bonds. Oscillations are characteristic of feedback-controlled systems and have been shown to correlate to changes in O_2 conditions in some bacterial fermentations. However, Buchanan *et al.* (1987) showed that the adenylate energy charge had no effect on aflatoxin production by *A. parasiticus*, which appeared to be due to a catabolite repression effect on TCA cycle enzyme activity via inactivation of the mitochondria.

On a time-scale of 1–10 hours, responses occur through induction and repression of protein synthesis. Changes between anaerobic and aerobic conditions can cause significant adaptations in microorganisms, for example, mitochondrial structural changes in yeasts (Visser *et al.*, 1990). Many enzymes in cells are regulated by O_2 availability, and the induction/repression of the synthesis of these may depend on concentrations of O_2 lower than the critical concentration required for respiration. Whether or not O_2 acts directly as an effector molecule has not been established; it may alter the redox state of specific redox couples or of an effector molecule within the cell.

The final level of metabolic response (which is more relevant to bacteria) is at the selection of mutants on a time-scale of many generations. Continuous selection occurs in chemostat cultures, where changes in DO cause strains of organisms more fitted to the predominant conditions.

INFLUENCE OF REDOX POTENTIAL ON MICROBIAL CULTURES

Redox studies of fermentations presuppose a very low DO concentration in the medium and O_2 acts as an oxidant. The redox potential (E_h) was proposed as a potential control in fermentation processes in which optimal product formation occurs at some point below full aerobiosis. E_h is defined by the Nernst equation, which is used to calculate electrode potentials or cell potentials for concentrations and partial pressures other than standard state values. Redox probes have been used to define what the E_h of a fermentation might be, but the result cannot be defined in terms of known electrochemical reactions. Observed correlations between metabolism and E_h changes could possibly be explained by changes in DO tension or substrate levels in the media. Direct fluorimetric measurements of NADH have shown that high frequency oscillations may occur, and the levels respond to changes in the O_2 supply, especially at low O_2 tensions. This type of experiment is considered to be a much better tool for observing the intracellular redox state and key redox couples (Harrison, 1972; Mukhopdhyay and Ghose, 1976). It has been considered that the overall NAD/

NADH ratio, or the redox potential of a cell, could be key to regulatory processes, but this has been dismissed as being of little value by Harrison (1972) in favour of responses of electron acceptors and metabolic products to changes in O_2.

OXYGEN AND YEAST METABOLISM

Yeasts (unicellular fungi of the class *Hemiascomycetae*) have been the subject of many studies regarding the effects of O_2 on their metabolism. Four effects have been determined which describe O_2-related physiological phenomena dependent on the species and on the sugar substrate.

The Pasteur effect describes the inhibition of fermentation in the presence of O_2, resulting in higher sugar uptake in 'fermenting cells' than respiring cells (Fiechter *et al.*, 1981). The effect occurs in resting cells of *Saccharomyces* species, and the increase in respiration rate caused by aerobiosis to increased ATP production is not due to increased respiratory capacity, but the loss of fermentative activity.

The Crabtree effect differs from the Pasteur effect because it is the substrate (eg glucose or fructose) which causes repression of respiratory activity under aerobic conditions in growing cells. It is divided into short-term and long-term effects. The long-term effect refers to oxido-reductive (or respiro-fermentative) growth during steady-state conditions in aerobic glucose-limited chemostat cultures where the specific growth rates are above D_{crit} (the switchpoint between purely oxidative and oxido-reductive yeast growth), and where ethanol production remains constant. The short-term Crabtree effect is a direct reaction to a 'glucose pulse', causing the immediate production of ethanol and acetate (Van der Aar *et al.*, 1990). Pyruvate decarboxylase has been shown to be activated after the 'glucose pulse' in response to increased pyruvate levels, and competes with the mitochondria for it (van Urk *et al.*, 1989). The Crabtree effect has been considered to be a stabilization, rather than a repression, of respiratory activity since the k_La remains at constant high levels at growth rates above D_{crit}.

The third effect is the Custer's effect, where fermentation is inhibited by the absence of O_2 in some yeasts, such as the genus *Brettanomyces* (Weusthuis *et al.*, 1994). It is considered a negative Pasteur effect and has been attributed to an initial shortage of NAD^+ brought about by the activity of redox systems in the cell after the addition of glucose. The effect can be reversed by the addition of various carbonyl compounds and re-oxidation of NADH to NAD^+ (enzymatically and with O_2, and through the respiratory chain) so that it can participate in fermentations. Gaunt *et al.* (1983) showed that this effect could be relieved with organic hydrogen receptors which restored the redox balance, although this cannot be explained in terms of the nicotinamide nucleotide pool.

The Kluyver effect is the fourth and least understood effect related to O_2 in yeasts, and occurs in over 40% of yeasts that ferment D-glucose anaerobically. It was originally defined by Sims and Barnett (1978) as occurring when certain yeasts utilize particular disaccharides aerobically, but not anaerobically, although under anaerobiosis the component monosaccharides were utilized. Weusthuis *et al.* (1994) redefined it as the inability to ferment certain disaccharides to ethanol or CO_2, even though the respiratory metabolism of the disaccharides and alcoholic fermentation of the component hexoses can occur. Accordingly, this definition suggests that the Kluyver

effect is not dependent on the O_2 concentration, but reflects an inability to metabolize the disaccharide fermentatively. Sims and Barnett (1978) concluded that the Kluyver effect resulted from the requirement for O_2 by sugar transport. In later work (Sims and Barnett, 1981), these authors showed that pyruvate decarboxylase activities were reduced when grown on the disaccharide. Other authors (Oura, 1974a,b; van Urk *et al.*, 1989) have implicated the role of this enzyme, which plays an essential role in glycolytic regulation under aerobic conditions. Along with its observed role in the short-term Crabtree effect (van Urk *et al.*, 1989), this enzyme can be considered an important switchpoint in mediating the various O_2 effects observed in yeasts. Weusthuis *et al.* (1994) indicated that there were three possible reasons why the Kluyver effect could be caused by differences in monosaccharide and disaccharide metabolism, sugar transport, disaccharide hydrolysis, and sugar-specific regulatory mechanisms which occur before the level of pyruvate (reiterating the conclusions of Sims and Barnett, 1978). The explanation proposed by Weusthuis *et al.* was that there might be a mechanism that tunes disaccharide uptake and hydrolysis in response to O_2 concentration or redox potential.

In studies unrelated to the four effects mentioned above, a series of papers by Bruver and Ball (Ball *et al.*, 1975a,b; Bruver *et al.*, 1975a,b) showed different, but feasible, means of regulation between cultures utilizing glucose or galactose as the respective carbon sources. Using an O_2 challenge to anaerobic cultures, different responses to step-down transfers and anaerobic to aerobic transitions were noted. It was proposed that adenine nucleotides controlled the metabolism of glucose cultures, and that the NAD^+/NADH level controlled galactose cultures; this hypothesis was based on the enzymes induced by these hexoses under anaerobic conditions. Phosphofructokinase was the rate-limiting step for glucose cultures since NAD^+/NADH cycling would not affect activity. The NAD^+/NADH cycling control in galactose cultures was considered to operate by failure to induce the alcohol dehydrogenase (ALDH) responsible for recycling, and/or by failure to induce pyruvate decarboxylase, so there would be no substrate for ALDH. The inability of galactose to induce glycolysis at the point of pyruvate to ethanol preconditioned the anaerobic cells, thus determining the subsequent response to O_2 challenge. In the glucose culture, an O_2 challenge resulted in a small amount of induced TCA activity (thus, the energy generating system was unaffected), whereas the galactose culture had pre-formed TCA activity facilitated by O_2, which out-competed the poorly-induced ethanol branch for pyruvate, resulting in decreased ethanol production. A 'control by induction' model for glucose regulation was proposed, rather than other catabolite repression models.

REGULATION OF GENE EXPRESSION IN YEASTS BY O_2

Many yeast genes are differentially expressed in response to O_2. During oxidative growth, more than 200 genes involved with respiratory functions and the control of oxidative damage are induced. At low O_2 tensions, hypoxic genes are expressed to allow the cell to utilize limiting O_2 more efficiently; these include cytochrome subunits and oxidases, plus desaturases involved in heme, sterol and fatty acid biosynthesis. Zitomer and Lowry (1992) published an extensive review of the mechanisms involved, which is summarized in the following paragraphs.

Heme is one molecule that plays a significant role in O_2 regulation. It is a prosthetic group in cytochromes and some O_2-binding proteins such as catalase, as well as acting as an intermediate in the signalling mechanism for O_2 levels in yeast cells. Heme biosynthesis has an essential requirement for O_2 at two enzyme-mediated steps, where it acts as an electron acceptor, one of which is the rate-limiting step for heme biosynthesis, and is therefore controlled by O_2 tension. The remaining pathway enzymes do not become functional until the presence of heme is detected.

There are two categories of heme-regulated genes: heme-activated and heme-repressed. Heme-activated genes fall into two main groups – those encoding respiratory functions (eg cytochrome units) and those encoding oxidative damage repair functions (eg catalase, Mn-superoxide dismutase). The heme-repressed genes are the hypoxic genes which function in the utilization of O_2 in electron transport, or in membrane or heme biosynthesis gene products that have no purpose in cells growing in complete absence of O_2.

Two distinct heme activation protein complexes have been identified: *HAP1* and the *HAP2/3/4* complex and at least four genes are activated by both. The *HAP1* protein binds to upstream activation sites (UASs) of a number of genes to activate transcription in the presence of heme. DNA binding is masked in the absence of heme. The *HAP2/3/4* complex responds to two stimuli for transcriptional activation to occur – heme and non-fermentable energy sources (eg lactate added as the carbon source instead of glucose). Some genes experiencing this glucose catabolite repression effect are activated by *HAP1* to overcome this. All three units are required for activation. *HAP4* is the transcriptional activation domain; *HAP2* is probably the DNA binding region. The *HAP2/3* heterodimer acts as a general transcription factor in the regulation in a variety of cellular functions and is believed to associate with different genes by using alternate *HAP4*-like sub-units, as the regulatory mechanism requires.

The *Rox1* repressor is responsible for heme repression of gene expression. It acts at two operator regions with the consensus sequence YYYATTGTTCT which lie between the TATA box and the UAS region, in a manner parallel to the activation of genes coding other translational components. Heme transcriptionally activates *Rox1* gene expression, partially due to *HAP1* and *HAP2/3/4*. However, it is not affected by catabolite repression like other genes regulated by this complex. Heme repression of gene expression by *Rox1* occurs only at transcription of the *Rox1* gene. Repression by *Rox1* requires two other proteins, *Tup1* and *Ssn6*, in a complex which serves as a general repressor, and it is thought the function of *Rox1* is to anchor the *Tup1-Ssn6* complex to specific genes targeted for repression since it contains a HMG box found in other sequence-specific binding proteins in its N terminus. There is also a C terminus domain that binds the *Tup1-Ssn6* complex (Deckert *et al.*, 1995).

Heme-independent O_2 regulation has also been found in yeasts in the form of two systems – mitochondrial gene regulation and anaerobic genes. Mitochondrial DNA encodes functions for its maintenance, and the sub-units of the oxidative phosphorylation apparatus – these genes are regulated by O_2. One example is *PET494*, a nucleus-encoded factor that works with two other factors (*PET122* and *PET54*) to recognize the 5' leader of CoxIII mRNA (cytochrome *c* oxidase sub-unit), and it is regulated at the level translation in an O_2-dependent, heme-independent manner.

Anaerobic genes (*ANB2–ANB15*), of which four are identified as 'early' and eleven as 'late' (ie they require at least three hours anaerobiosis), are expressed exclusively

in the absence of O_2. The early genes, *ANB13*, *ANB14* and *ANB15*, were heme and *Rox1* independent in action, as was the induction of four of the late genes. These anaerobic genes could be more sensitive to low O_2 levels than heme-repressed genes.

Oxygen and carbon metabolism

The effect of O_2 on carbon metabolism in hyphal fungi was investigated by Woodhead and Walker (1975), who studied the effects of aeration on glucose catabolism in *P. expansum*. Shake cultures of *P. expansum* showed considerable differences in enzyme activities and isozyme patterns compared to those observed in static cultures. In particular, the activities of two pentose phosphate shunt (PPS) enzymes, glucose-6-phosphate dehydrogenase (G6PD) and 6-phospho-gluconate dehydrogenase (6PGD), were elevated in shake cultures and this was reflected in the multiple bands present on isozyme gels. Both the enzyme activities and the number of isozymes decreased as the fungal metabolism entered idiophase later in the fermentation. By contrast, static cultures exhibited minimal PPS enzyme activities, with an extra isozyme not appearing until after 96 hours for both enzymes, leading to the assumption that, in static cultures, the PPS may not be significantly functional until idiophase. The polyketide secondary metabolites, patulin and citrinin, were produced in large amounts in the static cultures, which was consistent with other studies that have shown that polyketide production occurs when there is a lack of NADPH (used in reductive processes) produced by the PPS. In the presence of NADPH, acetyl CoA would normally be diverted into fatty acid biosynthesis but in these conditions would be utilized by the polyketide synthases. A fuller discussion of secondary metabolism and patulin biosynthesis is given later.

Glycolysis was important and occurred at the same rate in both shake and static cultures. The TCA cycle operated in both, but succinate dehydrogenase (SDH) activity was higher in shake cultures. The SDH isozymes behaved in a similar way to the PP shunt isozymes; in shake cultures these were greatest in number during tropophase, then decreased in idiophase, whereas in the static cultures these increased in number during idiophase.

Another important observation in these studies was that extracellular pectolytic enzyme activity was greater in shake cultures. The transamination activity linked to the TCA cycle increased in shake cultures which, together with the increased pectolytic activity, led to the conclusion that, in shake cultures, the production of pectolytic enzymes represented a diversion away from secondary metabolite production. The increased pectolytic enzyme activity could have been a response to more readily available and utilizable growth substrates for the fungus through the increased aeration levels.

Dijkema *et al.* (1985) investigated carbon metabolism in *A. nidulans* using ^{13}C NMR to monitor the levels of various polyols, TCA intermediates, amino acids, and phospholipids during growth on glycolytic (sucrose or sucrose/acetate) or gluconeogenic (acetate only) carbon sources under different conditions of aeration. Polyols are low molecular weight sugar alcohols, which form endogenous reserves that are continuously metabolized, even in the presence of exogenous substrates, with pool sizes and compositions varying according to stages in the fungal life cycle. Polyol resonances were found to dominate the sugar resonance region of the ^{13}C NMR

spectrum. The nature of the carbon source affected the pool sizes of the various polyols, and O_2 had an important effect on glycolytically grown cultures. O_2 stimulated flux through the PP shunt and glycolysis, the ratio of erythritol (from the PP shunt) to mannitol (glucose-derived) increased and there was an overflow of reduced metabolites at the PP shunt (eg erythritol and arabitol), and at the C_3 level of glycolysis (glycerol). Gluconeogenic substrates did not stimulate the PP shunt. The TCA and amino acid resonance region of the spectra showed pronounced glutamate and glutamine concentrations when vigorous aeration was employed, reflecting high TCA cycle activity. The results of both studies lead to the conclusion that O_2 plays a key role in stimulating PP shunt activities and the TCA cycle. Both these pathways are important in regulating other metabolic functions.

Aeration and fermentations

O_2 has been used as a factor to control the final product outcome of fermentations since it can influence the metabolic direction in an organism of interest. The following examples show how aeration can affect a target biochemical such as commercially important enzymes and antibiotics, as well as demonstrating the importance of such metabolic studies to improve fermentation efficiencies.

PRODUCTS OF PRIMARY (OR INTERMEDIARY) METABOLISM

Citric acid (a TCA cycle metabolite) production from *A. niger* is one example of a commercially important fermentation, and overproduction of this metabolite is achieved by using the specific O_2 uptake rate to control the intracellular ammonium (NH_4^+i) level (Kim *et al.*, 1991). Metabolic studies on this organism showed that NH_4^+i were potent regulators of phosphofructokinase (PFK) in glycolysis. Low levels of NH_4^+i resulted in increased citric acid production, especially once the culture was in idiophase and increases in NH_4^+i led to an increase in the specific O_2 uptake rate. The optimal value for NH_4^+i was 3–4.5 mM/g /cell since concentrations higher than this level limited citric acid production. It was proposed that sub-optimal concentrations of NH_4^+ ions would be necessary for cellular maintenance, and possibly counteract the feedback control of citrate at the PFK step, thus providing a mechanism for continued citric acid production.

A point to note when studying the role of a physical parameter such as aeration is that not only O_2 transfer processes are involved. Dissolved CO_2 concentration and its effects on citrate production by *A. niger* A60 was studied by McIntyre and McNeil (1997). CO_2 is believed to be inhibitory to the citric acid production process, and this would be especially relevant to such an industrial process. However, the role of CO_2 in the inhibition of growth and metabolism or the specific CO_2 species involved remains unclear. Below pH 4.0, the only major species present is $CO_{2(aq)}$. In this study, the growth of *A. niger* A60 was inhibited by continuous gassing at all concentrations studied and, with increased CO_2 concentration, the production of citric acid decreased.

The morphology of the organism also changed; the pellet diameter increased due to the formation of long hyphae around the pellet edge, which is unlike the short, stubby hyphae which are normally associated with citrate production. Alteration of substrate utilization, altered enzyme equilibria, specific effects on TCA and glyoxylate cycle

enzymes (through a change in pH or similar properties), and altered *A.niger* morphology were all considered as possible causes of these effects.

Production of itaconic acid (methylene succinic acid), another TCA cycle-derived metabolite, was also O_2 dependent. Itaconic acid is used in the formulation of acrylic resins and polymers, and in *A. terreus* is formed by the decarboxylation of *cis*-aconitate from the TCA cycle. Itaconic acid biosynthesis is dependent on media composition, and is strongly influenced by physico-chemical and biological factors. The fermentation pH must be strictly controlled at 1.8–1.9 and continuous aeration is required. Gyamerah (1995) showed that shaken submerged cultures of *A. terreus* were sensitive to the absence of O_2 for more than 5 minutes and also to metabolic inhibitors of ATP synthesis. This O_2 dependence required protein and ATP synthesis to maintain the fermentation, and possibly to maintain a normal physiological pH in the acidic fermentation despite ATPase having no effect on preventing acidification of the cytoplasm (the plasma membrane may have been impermeable to H^+ ions). The role of ATP might be the transport of itaconic acid from the cell?

Lazic *et al.* (1993) studied the effects of pH and aeration on extracellular dextran production by *Leuconostoc mesenteroides* in order to optimize conditions used industrially. Dextran is produced from sucrose by dextransucrase and occurs simultaneously with growth. The best yields of dextran and stability of the dextransucrase occurred when the O_2 transfer rate was equal to the maximum O_2 uptake rate of the organism. Under anaerobic conditions, the fructose and glucose formed by dextransucrase are utilized by the organism as an energy source and, at higher than optimal aeration, the glucose portion is utilized rapidly by the organism, reducing dextran yields.

Kojic acid is produced by *A. flavus* Link and has many industrial applications, including medical use as an anti-inflammatory drug and painkiller, as a precursor for flavour enhancers in the food industry, and cosmetically it is used as a whitening agent. The biosynthesis of kojic acid is believed to be mediated in part by enzymes of primary metabolism, such as glucose-6-phosphate dehydrogenase, hexokinase, and gluconate dehydrogenase, and an O_2 requirement is considered essential in the early stages of the fermentation since these enzymes are very important in glucose catabolism. Ariff *et al.* (1996) studied the optimum aeration requirements for this fermentation and showed that the fermentation required two phases: growth and production. Optimal kojic acid production occurred when the DO was controlled to 80% in growth phase, and to 30% in production phase when mycelial growth was reduced, provided no yeast extract or external N source was added, which resulted in the inhibition of production.

Pullulan is an extracellular polysaccharide from *Aureobasidium pullulans* that is formed from α-(1$\rightarrow$6)-linked maltotriosyl residues with an occasional random maltotetriosyl substitution. This polymer is used in a number of medical and industrial applications. Pullulan production by this fungus reduced with increasing agitation rates, and was shown to be due to increases in pO_2 in the medium and not increases in shear forces. The length of time that the fungus is exposed to low pO_2 conditions to improve production is believed to be critical; however, mechanisms for regulation were not described (Gibbs and Sevious, 1996).

ENZYME PRODUCTION

The effect of O_2 on growth and glucose oxidase production in *A. niger* was studied by Zetelaki and Vas (1968). Increases in both these parameters were observed when increases in agitation rate were applied to a 10 litre fermentor culture, with further improvements achieved by using pure O_2 instead of air. The viscosity of the culture increased with the increased concentration of mycelium that resulted, and decreased with the appearance of autolysis, which also occurred more rapidly in these cultures. Increases in sugar concentration in the more highly aerated cultures did not affect the growth and enzyme production rate, indicating that O_2 was the limiting, and therefore controlling, factor of the fermentation.

Another enzyme of interest produced by an *A. niger* strain is glucosyltransferase, an enzyme which produces iso-malto-oligosaccharides (IMOs), which are used as low calorie and non-carcinogenic sweeteners, and for the improvement of intestinal microflora by acting as a growth factor for Bifidobacteria. IMOs are produced by glucosyltransferase by the transfer of non-reducing D-glucose residues from a range of different donor substrates to suitable receptors. The results showed that maximum production of this enzyme occurred at an initial maltose concentration of 40 g/l, and at an agitation rate of 750 rpm and an aeration rate of 1 l/min in a 5 litre fermentor. This fermentation was dependent on a high DO concentration (Chen *et al.*, 1996).

Plant cell wall-degrading enzymes are important in processes such as kraft pulp bleaching and the degradation of resistant polymers such as lignin and related chlorinated compounds. Several studies have been undertaken to optimize the conditions for the production of these enzymes, and O_2 levels have been shown to play an important role in these processes. The cellulolytic enzymes of *Trichoderma reesei* QM9414 (a mutant for cellulolytic enzyme production), xylanase and endoglucanase, were studied along with growth and respiration under various agitation rates on two different substrates; lactose (for growth studies) and Avicel™ (a microcrystalline cellulose, for enzyme studies) (Lejeune and Baron, 1995). Pellet formation occurred with this fungus at all agitation levels for both substrates, but high agitation rates resulted in decreased enzyme production. Enzyme production in this fungus required growth to be limited and the hydrolysis of Avicel™ to have occurred.

Brandão Palma *et al.* (1996) studied the effects of agitation and aeration rate on xylanase production in *P. janthinellum*. This enzyme degrades xylans from the hemicellulose fraction of plant cell wall material. Xylanase synthesis was associated with growth and shown to be a function of k_La so that greater production occurred at low aeration and low agitation rates, and was more successful in shake flasks than in a stirred tank fermentor. This response to aeration and agitation was believed to occur because the process was sensitive to mechanical shear, but some agitation was deemed necessary to maintain medium homogeneity and avoid formation of large metabolically inactive pellets; however, large shearing forces could disrupt mycelial tissue and xylanase production. The inhibitory effect of O_2 on xylanase production was attributed to inhibition or inactivation of enzymes.

The formation and degradation of ligninolytic enzymes by *Phaenerochaete chrysosporium* was markedly affected by O_2. The production of extracellular proteases and polysaccharides was high with continuous oxygenation, and correlated to low levels and the fast decay of lignin and manganese peroxidases (LiP and MnP)

(Dosoretez *et al.*, 1990). The nature of the O_2 supply affected the patterns of appearance of these enzymes; a continuous or periodic supply of air maintained MnP supply but lignin peroxidase was undetectable, whereas a periodic supply of O_2 resulted in slower decay of LiP and a continuous supply of O_2 resulted in a rapid decrease in LiP activity.

ANTIBIOTIC PRODUCTION

The effects of O_2 on antibiotics produced by *Streptomyces* have been well studied, although there are few studies on eukaryotic fungi. One such study involved the use of miniature electrodes fitted into the sides of shake flask cultures of *Saccharopolyspora erythraea* and *Amycolatopsis orientalis* (Clark *et al.*, 1995). Erythromycin produced by *S. erythraea* paralleled biomass production, and was produced under both O_2-limitation and glucose-limitation, whereas in *Am. orientalis* vancomycin production required O_2 and occurred after growth slowed. The *S. erythraea* cultures also produced an unidentified red pigment in O_2-limited cultures; it was proposed that this was an 'overflow' metabolite of some type since the carbon source was still being assimilated.

Virgilo *et al.* (1964) showed that oxygenation was necessary for rifamycin production by *Streptomyces mediterranei* by measuring DO, O_2 diffusion rate, dry weight, viscosity, pH and sugar utilization for different impeller diameters, speeds and airflows. The period from 50 to 80 hours was identified as a critical time during the fermentation, during which high agitation speeds resulted in high antibiotic production since the O_2 demand of the organism was being met.

Rollins *et al.* (1988) studied cephamycin C production in *S. clavuligerus* and showed that the fermentation required near saturating levels of DO to maintain production without affecting growth rate. Reduced oxygenation possibly limited the availability of antibiotic precursors since precursors would be directed through the lysine pathway into proteins rather than to the ACV synthase, the first enzyme of the penicillin/cephamycin pathway. Similarly, amino acid pools may have been starved in such conditions, thus affecting amino acids involved in cephamycin C production; for example, methionine which stimulates ACV synthase, cyclase and expandase. This study was performed in 10 litre fermentors, and difficulties in controlling DO concentrations were expected in scaling-up the fermentation.

Positive effects of O_2 on antibiotic production have also been noted with *S. galilaeus* strains producing anthracyclines (Královcová and Vanek, 1979), and in colabomycin-producing *S. griseus* (Dick *et al.*, 1994). In *S. galilaeus*, the means of agitation had a considerable effect on the morphology and consequent biosynthetic activity. Pellets were formed when a reciprocating shaker was used, whereas a rotary shaker produced filamentous mycelium. Pellet formation resulted in decreased O_2 transfer and galirubin production. Colabomycin production by *S. griseus* was believed to be a result of the normal pathway of substrate utilization, involving glyceraldehyde-3-phosphate dehydrogenase, being bypassed via fructose-1-phosphate and glycerate, which may have caused an increase of precursors feeding into the cobalomycin polyketide synthase. Several studies have indicated the greater desirability of controlling DO and aeration parameters than mutant selection processes to industry.

O_2 can also affect the patterns of antibiotics produced by an organism. In another study on *S. clavuligerus* fermentations (Rollins *et al*, 1990), O_2 was shown to de-repress deacetoxy-cephalosporin C synthase (DAOCS), the enzyme responsible for converting penicillin N to cephamycin C. Under O_2-saturating conditions, DAOCS activity increased 2.3-fold compared to the penicillin ring-cyclizing enzyme, isopenicillin N synthase which increased only 1.3-fold. The quantitative ratio of cephamycin C production to total antibiotic production gave a measure of the comparative efficiencies of the two enzyme systems. O_2 also affected the time of appearance of cephamycin C. The mechanism(s) for de-repression is unknown.

The pattern of antibiotic production by *S. fradiae* was also affected by O_2. The organism used was a stable mutant producer strain (NRRL 2702) which produced tylosin, a macrolide antibiotic. At high DO levels and high aeration rates, an additional macrolide antibiotic, macrocin was synthesized. Macrocin is produced on the biosynthetic pathway leading to tylosin, and it was concluded that production was due to the enhancement of enzyme activity by the conditions rather than repression or inhibition of the enzyme responsible for converting it to tylosin. It was also inferred that co-factors and precursors were not limiting since this appeared to be the rate-limiting step of this mutant (Chen and Wilde, 1991).

One bacterial fermentation showed that O_2 can interfere with antibiotic production. *Bacillus brevis* required O_2 for growth to occur, but gramicidin S production was inactivated by its presence. The gramicidin S synthetase complex (which catalyzes non-ribosomal protein production) required stabilization by N_2, since the rapid disappearance of this enzyme at the start of stationary phase was attributed to O_2 (Friebel and Demain, 1977).

Secondary metabolism: an introduction

Secondary metabolism refers to a series of anabolic processes that can occur within an organism but are not considered to be essential for the organism's survival and growth. The products of secondary metabolism (idiolytes), can occur uniquely in a single strain or species, in closely-related members of a single genus, or may be found sporadically in a limited number of evolutionarily-unrelated species (Campbell, 1983). Secondary metabolites usually accumulate in particular organs or tissues, mainly in plants and microorganisms (Haslam, 1986), and include a wide range of chemical structures. These include amino sugars, quinones, coumarins, epoxides, ergot alkaloids, glutarimides, glycosides, indole derivatives, lactones, macrolides, naphthalenes, nucleosides, peptides, phenazines, polyacetylenes, polyenes, pyrroles, quinolines, terpenoids, and tetracyclines (Martin and Demain, 1980). Some of these structures may include biologically unusual chemical groups such as covalently-bound chlorine and bromine; nitro, nitroso, isonitrilo, diazo and hydroxamic groups; phosphono, phosphino, phosphonamido; unusual ring systems such as β-lactams, tropolones, dioxopiperazines, macrolides, xanthones, isochromaquinones (Zahner, 1979).

The biosynthesis of secondary metabolites is generally directed by organized sets of genes associated with special regulatory mechanisms that control both the timing and level of gene expression (Vining, 1992). These control mechanisms are well integrated with the physiology of the producing organisms. Many secondary

metabolites have been found to be growth-inhibitory or toxic to other organisms, or the cause of allelopathy in plants (ie chemicals which inhibit the growth or reproduction of other plant species with which the producer is competing) (Haslam, 1986). Possible roles for these and their significance are discussed later in this section. Secondary metabolites have been referred to as idiolytes, shunt metabolites, special metabolites or 'chemists' compounds' (Campbell, 1983).

By contrast, primary or intermediary metabolism (primary metabolites are sometimes called 'biochemists' compounds') is concerned with the pathways for energy production and the central processes for cellular survival. Primary metabolism involves an interconnected series of enzyme-mediated catabolic, amphibolic and anabolic pathways which provide biosynthetic intermediates and energy, and convert biosynthetic precursors into essential macromolecules by processes common to all living things (Martin and Demain, 1980). A series of kinetically controlled enzyme reactions link primary metabolism, and these normally function with very low pool concentrations of primary metabolites, which, therefore, are unlikely to accumulate. Thus, primary processes tend to be governed by substrate levels, whereas secondary processes are limited by their enzymes (Haslam, 1986).

Howsoever, the boundary between primary and secondary metabolism is ill-defined. There are compounds that appear to be essential metabolites, but there is no conclusive evidence to support any of their proposed cellular functions. This may simply reflect a lack of knowledge of the biological function of secondary metabolism. Many secondary metabolites have acquired a physiological role in the inter- and intra-specific interactions of organisms; for example, pheromones and phytoalexins. There are also some primary metabolites which can be classed as secondary metabolites because of the nature of their production; for example, the overproduction of citrate by certain *Aspergillus* species appears to have no cellular function and, therefore, may be classed as a secondary metabolic event (Garraway and Evans, 1984). There are several theories which attempt to explain the '*raison d'être*' of secondary metabolism and it is generally assumed that secondary metabolism must have been of some selective advantage to the organism during its evolution. The following is summarized from Haslam (1986).

The first category for theories concerning the existence of secondary metabolism is that at some stage in the life of the organism the secondary metabolites had, or still have, some functional or metabolic role. A second category view secondary metabolites as waste or detoxification products. The third sees secondary metabolism as a measure of the fitness of an organism to survive since compounds are produced which may attract or repel other organisms as part of that organism's strategy for survival. The final category is that secondary metabolism may allow organisms to adjust to changing circumstances. Thus, the synthesis of enzymes for secondary metabolism permits the network of primary enzymes to continue to function until circumstances allow renewed metabolic activity and growth. This implies that it is the processes of secondary metabolism, rather than the products, which are important, and that these secondary metabolites have subsequently acquired a functional role.

Several ideas have been put forward based on the final hypothesis. Woodruff (1966) proposed that secondary metabolites are 'shunt' metabolites which serve to reduce abnormal concentrations of normal cellular compounds (and which may be toxic at certain levels). Shunt metabolites form when other pathways for that

intermediate are wholly or partially closed when growth ceases (Bu'Lock, 1961) and, therefore, high pool levels of intermediary metabolites accumulate. Shunt metabolism results in the modification of normal pool metabolites that tend to involve condensations and polymerizations. Here, the processes of secondary metabolism are more essential than the products because secondary metabolism allows the uptake of nutrients to continue; thus depriving competing organisms of nutrients not already exhausted. Bu'Lock and Powell (1965) suggested that secondary metabolism serves to maintain basic metabolism in circumstances when its normal substrates, through depletion of nutrients, can no longer be exploited for normal cellular growth and replication. The advantage to the organism occurs with the rapid renewal of growth once favourable conditions return. Metabolic intermediates normally present at low concentrations accumulate, leading to the induced formation of new enzymes to relieve the pressure on key metabolic intermediates. Another idea is that secondary metabolites are waste products of general metabolism in phylogenetically less-advanced organisms (Haslam, 1986).

The sophistication of the biosynthesis, regulation and genetic organization of secondary metabolism leads to the assumption that it must be beneficial to the organism, and that it would have been eliminated if it had not been maintained by selection. This rationale overcomes the argument that secondary metabolites are 'metabolic waste' and, instead, detoxification, storage, metabolic idling or the shunting of metabolic overloads provide the overall rationale for the process (Vining, 1992). Some plausible hypotheses relate to signalling, differentiation, sequestering nutrients from the environment, mediating ecological interactions, and facilitating biochemical evolution. However, the majority of secondary metabolites are biologically active compounds with two principal themes for their function; *either* to serve a purpose within the producing species such as mediation of growth, reproduction or differentiation, *or* to act on targets external to the producer organism. Examples of the latter include the production of antibiotics, insecticides, herbicides and mycotoxins, some of which appear unnecessary to the producer organism (Vining, 1992). With respect to the role of antibiotics, Woodruff (1966) suggested that they do not confer a survival advantage if one considers that antibiotic production is the normal situation in microbial growth due to limitations of the environment. However, in the context of microorganisms living with, and competing against, each other in nature, antibiotics are not produced to the same extent.

As well as there being several hypotheses for the role(s) of secondary metabolism, there are also three categories of theories to account for the evolution of such biosynthetic pathways. The first group includes subtractive theories based on the idea that ancestrally inefficient, but potentially versatile, organisms evolved more discrete pathways by the development of increased specificity. Opposing these are the accumulative theories in which extant biochemical pathways are presumed to have arisen from simpler, ancestral pathways. Theories of retrograde evolution make up the third group where a backward-evolving pathway recruited new enzymes in reverse order (Haslam 1986). The primitive enzymes of ancestral cells probably possessed very broad specificities in order to exploit a range of resources prior to nitrogen being introduced into metabolism, since the main energy producing pathways of the cell, glycolysis and the TCA cycle, do not directly involve nitrogenous compounds. Retro-evolution, involving the antecedents of secondary metabolism,

could be responsible for shaping the metabolic systems and pathways responsible for their biosynthesis; nevertheless, all secondary metabolic pathways originate from a precursor in the primary metabolic network (Vining, 1992).

Vining (1992) also discussed theories based on the idea that individual secondary metabolic pathways developed as a result of a chance doubling, and subsequent mutation, of a gene that originally directed the synthesis of a primary metabolic enzyme. Subsequently, the altered copy acted on the substrate to give an abnormal product(s), which became useful to the organism after spontaneous chemical change, or by resident enzymes of relaxed specificity. Random modifications may have occurred through several stages before the chance formation of a beneficial product resulted in a strain possessing a selective advantage. Further gradual improvement of this pathway would have consolidated these benefits and ensured maintenance of the genetic information. Thus, secondary metabolism could have developed stepwise, in a forward sequence, from a pre-existing primary network that provided the essential metabolic needs of the organism.

Zahner *et al.* (1983) proposed the concept of a 'biochemical playground' where secondary metabolism operates in parallel with the five primary cellular events of intermediary metabolism, regulation, transport, differentiation and morphogenesis, so providing an opportunity for continual biochemical evolution. The existence of this 'playground' requires the supply of surplus precursors and energy from intermediary metabolism, whilst secondary metabolism allows the evolution of biochemical pathways to proceed in all directions, provided the new metabolites are not toxic to the producer at the time of their appearance. Continuous evolution of pathways in secondary metabolism occasionally leads to compounds which offer an advantage to the producer organism and, once developed, become a part of primary metabolism. The functional equivalence of structurally different compounds is an important feature of the postulated 'playground'. Once a useful secondary metabolic pathway has been developed, it can be utilized by other organisms. The symbiont theory of eukaryotic evolution is an example of this where pre-existing information was utilized because it gave an advantage over the *de novo* synthesis of genetic information. The transfer of genetic material occurs readily through symbiont-host and parasite-host type relationships, or through close contact of organisms in common habitats such as soil. Thus, communication of useful information by genetic transfer amongst different organisms becomes possible.

The genetics of secondary and primary pathways has been compared. An example is the similarity of polyketide biosynthesis to that of fatty acids, with the main differences being the partial or complete absence of reactions to reduce the β-keto group after chain extension, and the larger variety of acyl precursors incorporated (Vining, 1992). Similarities in the sequence of amino acid in the proteins that catalyze these reactions infer a common evolutionary origin. However, the lack of similarity between polyketide synthase and fatty acid synthase from the same organism does not agree with the gene duplication and subsequent evolution theory. Another example is that of the β-lactams; ACV define synthase catalyzes the condensation of the three amino acids, α-aminoadipic acid, cysteine and valine, in a manner which resembles the polypeptide synthases from *Bacilli*. Nucleotide sequencing confirmed an evolutionary relationship, but this holds no similarity to ribosomal polypeptide synthesis in primary metabolism and, therefore, is not a modification of existing

primary metabolic pathway genes. Other enzymes involved in β-lactam biosynthesis have similar traceable origins and similarities amongst themselves, or related processes, in the pathway. It is also inferred that, in this case, entire gene clusters have been transferred because of the similarities of sequences, the lack of introns, and the persistence of gene clusters, even though arrangements within these have subsequently changed around (Vining, 1992).

Industrially, secondary metabolism is a useful concept for predicting process characteristics since certain physiological events coincide with the initiation of product formation (Bushell, 1989). Three types of processes have been described (although these descriptions are now considered simplistic and outdated):

Type I: formation of growth-associated products that are involved in the formation of daughter cells or energy metabolism associated with undifferentiated growth.

Type II: products formed from substrates involved in primary metabolism but via alternative pathways.

Type III: product formation occurs at an entirely different time to primary metabolism and is often derived from amphibolic pathways (bi-directional), rather than from catabolism.

Since secondary metabolism occurs in response to the concentrations and assimilation kinetics of many of the principal culture nutrients, switch-on may overlap with the growth phase if a specific regulatory nutrient is depleted. If the nature of this regulation is understood, then secondary metabolism can be predicted and also manipulated; however, this varies for different organisms and biosynthetic processes.

Borrow *et al.* (1961, 1964) defined five distinct phases of growth in *Gibberella fujikuroi* when producing gibberellic acid. This was achieved by varying concentrations of the key nutrients glucose, N, P and Mg, in all possible sequences, so that all four were exhausted at a selected final dry weight, together with studies of other factors in the fermentation such as pH (initial and at nutrient exhaustion), agitation rate and airflow, and inoculum size. The productivity decreased with increasing glucose concentration, and the maximal amount produced was proportional to the initial N provided.

The first 'balanced phase of growth' was a period when rapid growth and nutrient uptake extended from the start of growth until the first nutrient was exhausted (glucose and N had the greatest effects). Here, an apparently preferred constant form of mycelium was being produced in the nutritionally unlimited conditions occurring at the initial specific growth rate until some change affected metabolism. Growth in the balanced phase was defined to fit an exponential growth model. At least five forms of metabolism can follow the first period of exponential growth in N-limited fermentations: storage, exponential, decelerating, step and linear. Linear growth may, in turn, be followed by a form of decelerating growth, while the storage phase occurred after N exhaustion, irrespective of the form of growth preceding this event. Linear growth is evidence of a response to O_2 restriction: increased mycelial growth results in decreased turbulence and rate of O_2 supply rate, whilst the demand for O_2 increases. Therefore, the onset of linear growth is the point at which demand exceeds supply. The rate at which exponential growth changed to linear growth was greater the higher the rate of agitation.

In the first study, the second 'transition phase' followed the balanced phase and was characterized by continued increases in the mycelial lipid and carbohydrate content after exhaustion of the limiting nutrient. Proliferation of the fungus still occurred, but the mycelial composition was changing from that observed during the growth phase. This transition phase continued until reserves ceased to be utilized, by which time the 'storage phase' began. During storage phase, nutrient uptake, except for glucose, ceased, whilst carbon and other reserves accumulated in cells whose cellular composition was being reorganized after proliferation had ceased. Dry weight reached its maximum value during this phase. The 'maintenance phase' occurred when external, or internal, carbon sources were metabolized by virtually unchanging cellular material and continued until no utilizable carbon remained. During the storage and maintenance phases, the amount of living matter remained virtually constant, whilst the total dry weight continued to increase during storage phase so turbulence may continue to decrease. At this point, the 'terminal phase' of mycelial breakdown commenced. This autolytic phase was characterized by increased vacuolation and loss of cell contents, and the return of NH_4^+, P, Mg, and K to the medium, as well as an increase in medium pH to greater than 8.

Nowadays, Borrow's balanced growth phase is analogous to tropophase, and the storage and maintenance phases are considered part of idiophase.

The regulation of secondary biosynthesis has been studied in *G. fujikuroi* grown in batch and chemostat culture, and the major features of batch culture development were shown to relate to the degree of limiting substrate depletion and the corresponding growth rate (Bu'Lock *et al.*, 1974). The major features of secondary metabolism in this organism were the production of bikaverin (a red, polyketide, anti-protozoal quinone), gibberellin (terpenoid), and mycelial carbohydrate accumulation (which occurred with declining growth rate and is therefore believed to be a non-vegetative growth process). Six phases regarding growth and products were described for the batch cultures of this organism. Bikaverin was produced earlier than gibberellin, but both were in response to declining N concentrations. Thus, production was controlled by a common mechanism of growth-linked suppression to which the two processes had differing sensitivities. Alterations to the dilution rates in chemostat cultures also resulted in the differential synthesis of both compounds at different rates, gibberellins being at a lower rate than the bikaverins. Replacement cultures were also performed with the mycelium being replaced onto N-free medium; this resulted in bikaverin production but, when glycine was re-introduced, production was stopped. Since this was maintained only when RNA and protein synthesis were not inhibited, it appears the turnover of synthetase components was involved in the rate-limiting mechanism. Gibberellin synthesis was under the same type of control, except exerted down at a lower level of limiting nutrient and a corresponding lower growth rate. Exhaustion of the N source was believed to be the means by which this growth-linked suppression mechanism operated.

However, studies by Grootwassink and Gaucher (1980) showed that, in a heterogeneous cell population, overlapping of the growth and production phases can be observed. Their studies on the production of patulin by *P. urticae* showed that patulin biosynthesis required a carbon source, but it was the depletion of N that triggered the production of this polyketide. They showed also that the secondary pathway enzymes appeared sequentially, prior to patulin production, and were synthesized *de novo*.

Secondary metabolism can be described as biochemical differentiation that may accompany morphological differentiation in a variety of fungi. It is a response to conditions not permitting further cell multiplication. The morphological response is to form specialized structures (eg conidia, sclerotia) to ensure prolonged survival, and resumption of growth at a later time is a more long-term process. However, there is evidence to indicate that the two processes are interrelated.

Differentiation can be described as the stable development of an altered structure or function, and in fungi this occurs at the end of active growth phase, resulting in a morphologically diverse array of structures. It is irreversible and excludes transient changes referred to as adaptations. Two mechanisms of gene expression are involved. In the short term, the timing at which initiation of differentiation occurs, and the determination of pathways is dictated by nutrient conditions. In the long term, changes in enzyme composition of the cell are responsible for regulating differentiation mechanisms (by genetic control) (Martin and Demain, 1978).

The relationship between secondary metabolism and differentiation in fungi has been alluded to by a number of authors. Woodhead and Walker (1975) suggested there might be a relationship between 6-methylsalicylic acid (6-MSA) and the initiation of sporulation in *P. expansum*, but this required further investigation. Guzman-de-Pena and Ruiz-Herrera (1997) showed that diaminobutanone, which competitively inhibited ornithine decarboxylase, repressed spore germination. If added after spore germination occurred, it completely blocked both sporulation and aflatoxin biosynthesis. Bennett (1983) reviewed both secondary metabolism and sporulation, noting that sporulation, like secondary metabolism, was usually induced by a decrease in available nutrients. Both processes start after active growth has stopped and tend to have narrower ranges of trace metal, pH and temperature requirements than growth. Both also occur in distinct families of morphology (differentiation) and chemistry (secondary metabolism). Although analogous, the two processes are often viewed separately and not as part of a single differentiation process.

The nature of the growth medium will dictate the growth form, which, in turn, dictates the growth kinetics of a fungal culture. Liquid culture provides a more homogeneous environment than a solid medium since nutrients are available via diffusion (Martin and Demain, 1978). O_2 is the exception because of its low solubility in water, and can be limiting unless high transfer levels are maintained. The implications of agitation and O_2 transfer have been discussed earlier.

Earlier research into secondary metabolism tended to focus on the discovery, identification and production of biologically active compounds, and early research had an organic chemistry emphasis. Not only were compounds identified, but also syntheses to attempt to parallel the biosynthetic capabilities of producer organisms; difficult because of the stereo-chemical constraints of many of these compounds. The focus then moved as to how the organism produced a secondary metabolite from simple precursors, which supplied carbon, hydrogen, oxygen and nitrogen, to the metabolite in question. As a result of such research, three main groups of secondary metabolites were established: polyketides (derived from repeating acetate units), terpene derivatives (via the isoprenoid pathway), and amino acid derivatives (for example, from aromatic amino acids) (Campbell, 1983).

Major pathways of secondary metabolism: an overview

(Key references: Bu'Lock, 1961; Garraway and Evans, 1984)

ISOPRENOIDS

Terpenes and sterols are the two main products of this pathway that involve the head-to-tail condensation of C_5 isoprenoid units. These isoprenes are formed via the mevalonic acid pathway from acetyl CoA, which is converted to hydroxy-methyl-glutaryl CoA (HMG CoA), then to mevalonic acid, and eventually to isopentenyl phosphate (IPP) and dimethylallyl phosphate (DMAP); the 'C5 building blocks' which undergo further condensation into larger units. Examples of these include ergosterol, a ubiquitous fungal steroid, carotenoids, and the gibberellin plant growth regulators. In terms of the variety of structures, the isoprenoids are less important in fungi than in higher plants in which terpenes are the most abundant natural products.

POLYKETIDES

This class of compounds is characteristic of fungi and, in particular, the Deuteromycetes. Acetyl CoA condenses with repeating units of malonyl CoA (with the loss of CO_2) to form chains of polyketide precursors (eg decaketide). These then cyclize and may undergo further reactions, such as oxidation, reduction, substitution, ring opening, ring coupling and rearrangements, to form a vast array of polyketide products; for example, patulin and the aflatoxins. The processes by which these are produced are very similar to fatty acid biosynthesis but lack the systematic dehydration and reduction reactions of this pathway.

SHIKIMATE-CHORISMATE

This pathway produces the aromatic amino acids, which subsequently can be converted to secondary metabolites such as cinnamic acids (for example, protocatechuic acid), indoles (for example, echinulin), ergosterine or to lysergic acid through reaction with other compounds. Phosphoenol pyruvate (PEP) (from glycolysis) and erythrose-4-P (from the PPS) lead to shikimic acid, then chorismic acid and, finally, the production of the three aromatic amino acids, phenylalanine, tyrosine and tryptophan. This pathway is responsible for the production of many compounds based upon the phenylpropane structure in a wide range of plant products. It is of lesser importance in microorganisms.

NON-AROMATIC AMINO ACIDS

Examples of areas of biosynthesis include the β-lactam antibiotics, such as the penicillins and cephalosporins. These are formed initially from the condensation of the three amino acids, valine, cysteine and α-aminoadipate. Other examples of this type of biosynthesis include the non-ribosomal production of gramicidin S, a cyclic peptide produced by *Bacillus* species, other cyclic peptides such as phallotoxins, and the production of muscarine from glucose by *Amanita muscaria*. Many antibiotics are

produced from amino acids and peptides. Other examples of fungal metabolites include the diketopiperazines, gliotoxin and the *Amanita* toxins.

MISCELLANEOUS COMPOUNDS

Secondary metabolites may also consist of other structural groupings. There are simple organic acids that include a number of simple aliphatic acids not directly related to lipids. Examples include accumulating intermediates of general metabolism, such as citric, fumaric and oxalic acids, also modifications or derivatives of general metabolites, such as itaconic acid (from *cis*-aconitate).

Fatty acid derivatives constitute another structural group. These include the polyacetylenes from Basidiomycetes, polyene chains, branched chain analogues, propionate-derived C_3 analogues, and the aglycones of macrolides, such as erythromycin and polyene antibiotics like lagosin. Derivatives of sugars form another biosynthetic category which includes simple sugar acids and alcohols that are accumulated by some microorganisms (eg kojic acid); neomycins (derived from ribose), and unusual sugars, such as amino and branched chain sugars (for example, desosamine in several macrolides, cordycepose in cordycepin).

Purine and pyrimidine derivatives also occur in secondary metabolism, although little is known about the role of these nitrogen compounds in secondary metabolism. Finally, many compounds are of mixed origin, synthesized from two or more of the classes of compounds outlined above.

Enzymes of secondary metabolism

Significant differences have been observed in the properties of the enzymes of primary and secondary metabolism. Secondary enzymes tend to show broader substrate specificity and, therefore, are less sensitive to structural variations in the substrate. This feature has been exploited in precursor-directed biosynthesis; for example, with penicillins where altered substrates are fed to a producer organism or system, resulting in unique structural features that may improve the pharmacological properties. Usually, the enzymes of secondary metabolism are not as efficient as those of primary metabolism since the intracellular concentrations are lower and the reactions are catalyzed relatively slowly. An example of this is *m*-hydroxybenzyl alcohol dehydrogenase in the patulin biosynthetic pathway in *P. urticae*, for which the reverse reaction is thermodynamically favoured (Forrester and Gaucher, 1972). The enzymes of secondary metabolism may form a branching network of alternative pathways for which acetyl CoA is an important precursor since this compound is the branch-point to terpenes, steroids, fatty acids and their metabolites, and polyketides. The regulation of secondary metabolism is still not well understood. Initiation has been shown to be linked with morphological changes, changes in enzyme and substrate concentrations, and nutrient and external stresses (Haslam, 1986). A more detailed review of the regulation of secondary metabolism is given earlier.

Despite the diversity of products, analogies may be drawn concerning the nature of the reactions of secondary metabolism. Many secondary metabolic pathways involve polymerization processes; examples include the production of peptides from amino acids by synthases involved in non-ribosomal peptide synthesis and, secondly, the

condensation of C_2 units (from malonyl CoA) leading to the synthesis of polyketides, macrolides and polyethers. The acyl and amino acid units involved in these polymerizations are both bound to the enzyme/synthase as thioesters, with at least two types of sulphydryl group on the enzyme; for example, there is both a peripheral and a central sulphydryl on 6-MSA synthase (Behal, 1986).

The synthase complexes involved in the first steps of the pathways of secondary biosynthesis can be dissociated into functional sub-units, although the biosynthetic activity of the complex is lost. These complexes are important to the cell since they eliminate the necessity for intermediates to diffuse from one enzyme to another, which would limit the rate of the process. They have been found in subcellular particles, but this may be an artifact of the methods used to isolate them. The location of the secondary enzymes in cells may be important for the resistance mechanisms of the microorganism to its own products, so it is to be expected that these are compartmented (Behal, 1986).

Although it had been assumed that the limiting factor in secondary metabolite production was the availability of biosynthetic units in the cells, studies have shown that secondary metabolism is also dependent on the activity of secondary enzymes in the cells (Behal, 1986).

Secondary metabolism: gene expression and organization

The genes for secondary metabolism in bacteria are found on the chromosome or on plasmids, and in eukaryotic organisms on different chromosomes. Clusters of biosynthetic genes have been found, and linkages commonly occur between the genes for antibiotic resistance and biosynthesis. Sub-clustering, for example in genes for the production of tetraenomycin and actinorhodin, may act as a control mechanism for the timing and sequential appearance of each enzyme in the pathway. This is achieved in prokaryotes by coupling transcription and translation so that the product of a previous enzyme may be acted upon. However, in fungi, the sequential formation of enzymes has been shown to be an efficient way to ensure that the spatial constraints for the occurrence of sequential reactions is by the formation of multi-catalytic site proteins or multi-enzyme complexes, on to which the intermediates are bound, thus ensuring their availability for reaction. In eukaryotic organisms, compartmentation of enzymes can also ensure the spatial availability of reaction products to the appropriate enzymes.

Coordination of the expression of biosynthetic and resistance genes is necessary in order to avoid suicide of the producer organism and, therefore, induction and repression mechanisms are involved. In organisms such as *S. griseus* and *S. rimosus*, *tetA* and *tetB* resistance genes are induced by tetracycline, the toxic end product. For the production of streptomycin by *S. griseus*, positive and negative controls affect the production of the SPH protein, which is induced by streptomycin (the final product). An open reading frame, ORF, downstream of *strA*, is required for induction and is likely to encode a repressor protein, whilst the *strR* gene also positively regulates the *strA* gene. Two or more resistance mechanisms can be found in some *Streptomyces* spp., with at least one linked to the cluster of biosynthetic genes. Another case of resistance regulation is where the resistance gene catalyzes a reaction in the biosynthetic pathway so that the resistance and biosynthetic genes are under the same controlling

mechanism. The A-factor of *Streptomyces* is involved in the induction of resistance, as well as biosynthesis.

Some bacteria (*E. coli* and *B. subtilis*) have been shown to utilize a mechanism involving the production of alternative sigma factors or ancillary proteins that are able to alter the specificity of RNA polymerase. Promoters for antibiotic resistance and biosynthesis genes have been cloned, and show no resemblance to the normal consensus promoter sequences. It is possible that most secondary metabolism gene promoters have a unique structure and, therefore, are only able to be expressed under specific conditions leading to secondary metabolism. Evidence has shown that multiple sigma factors exist, but the physiological significance of different RNA polymerases under different conditions is not clear. Ancillary regulatory proteins may also play an important role in controlling differential gene expression in secondary biosynthesis. A model has been described for the *spoA* dependent inactivation of the *B. subtilis* gene *abrB*; this proposes an ancillary protein to be a negative regulator of *tycA* transcription (ie a secondary metabolism gene with a promoter resembling the canonical structure of the main class of promoters found in this species). The nutritionally regulated inactivation of a*brB* would result in the late induction of *tycA* synthesis in secondary metabolism (Martin and Liras, 1989).

Regulatory mechanisms

The production of secondary metabolites is delayed until idiophase by a variety of mechanisms, which is advantageous to antibiotic producing organisms since they may be sensitive to their product(s) during the growth phase. It is also advantageous in nutritionally poor conditions (when secondary metabolites are produced) for the antibiotic/toxin producer to possess the potential to compete with other micro-organisms.

ENZYME REPRESSION AND INHIBITION

(Key reference: Martin and Demain, 1980)

The initiation of secondary metabolite biosynthesis can be controlled by repression (prevention of gene transcription) or by inhibition of biosynthetic enzymes. Since expression of biosynthesis does not occur at high growth rates, it can be assumed that the synthases are not formed, or are inhibited. The regulatory process does not have to be exerted at a single level, and it is possible to find both inhibition and repression involved. For example, the onset of biosynthesis could be repressed until a specific nutrient is exhausted, while synthesis of compounds could be inhibited by a controlling factor until after the synthase has been formed. Late enzyme formation has been noted in the production of some antibiotics, so control may have occurred by interference with gene transcription. An example of this is amidinotransferase and streptidine kinase in streptomycin biosynthesis, which are both repressed during the growth phase. The amidinotransferase is synthesized *de novo* since the process can be inhibited by chloramphenicol.

The delay of the onset of secondary metabolite biosynthesis can also be due to pre-existing enzymes, which are unable to operate in the presence of an inhibitor.

β-lactam antibiotic production by *Cephalosporium acremonium* occurs after the growth phase has ended, but is not inhibited by protein synthesis inhibitors.

Feedback inhibition (also called end product or allosteric inhibition) allows a cell to monitor the production of a particular compound so that its synthesis may be switched off when a certain level is reached, or switched on if concentrations drop too low. The final metabolite of a biochemical sequence inhibits the action of an enzyme (usually the first) of that sequence by binding to it at a site separate to the substrate binding site in such a way that substrate-binding is impaired. This is a rapid mechanism for the maintenance of a constant concentration of low molecular weight metabolites (Demain, 1966). Repression occurs when a derivative of the end product, possibly the product in combination with an aporepressor such as a protein, inhibits the formation of enzymes in a pathway so as to control the capacity for synthesis of certain proteins (Demain, 1966, 1968). Chloramphenicol, cycloheximide and mycophenolic acid are examples of secondary metabolites where feedback inhibits their own production, although the molecular mechanism is not understood. The synthesis-inhibiting level in a producer strain is often similar to the production level; for example, mycophenolic acid causes 68% inhibition of SAM:demethyl-mycophenolic acid-*O*-methyltransferase, the final step in its synthesis (Martin and Demain, 1980).

Inhibition can also occur in a pathway where there is a common branch between primary metabolite formation and secondary metabolism, where the primary end product feeds back to inhibit the common part of the pathway. One example of this is the inhibition of penicillin production in *P. chrysogenum* by lysine (Martin and Demain, 1980).

Most end products are formed via branched pathways, and mechanisms have been identified for the prevention of interference by one end product on another derived from the same metabolic pathway so that the excess of one does not impact negatively on the others (Demain, 1966). The production of multiple enzymes or isozymes is one means by which this can be achieved. Each isozyme catalyzes the same reaction but is controlled by a different end product. Multivalent (or concerted) feedback requires all end products to be present in excess in order for repression or inhibition to occur. Finally, cumulative feedback occurs when each end product can provide only limited inhibition alone, but with increases in inhibitory effects when combinations of end products are involved. Complete repression/inhibition requires all the end products to be present.

MECHANISMS TO CONTROL INITIATION OF BIOSYNTHESIS

Two models describe possible mechanisms for controlling the start of secondary metabolism. The first involves a small molecule that acts as a co-repressor, or inhibitor, which inhibits/ represses synthases, and which must be depleted in order for synthesis to occur. In the second, an inducer, or an activator, must be synthesized to initiate biosynthesis.

Carbon catabolite repression is the repression of synthesis of enzymes by the catabolic products of a rapidly assimilated carbon source, commonly glucose (Demain, 1968). For many fermentations, oligo- or polysaccharides are better carbon sources than monosaccharides, and it is necessary to add a more slowly utilized carbon source to a medium containing glucose for its growth phase. The glucose is utilized first and,

in the absence of any production, this is followed by the second carbon source for antibiotic/secondary metabolite synthesis. Other carbon sources can also be involved; for example, citrate suppresses novobiocin production, but production will occur when glucose is used in the secondary phase. Since many of the preferred carbon sources are metabolized through the same intermediates as glucose, it appears that a precursor effect is not operating.

A molecular mechanism may also be related to the control of growth rate during secondary metabolism. A slow carbon feed results in a slow growth rate, therefore eliminating interference by glucose or similar. In a few cases, glucose has been shown to repress a known biosynthetic enzyme, an example being phenoxazinone synthase (PHS), the enzyme forming the phenoxazinone nucleus of actinomycin in actinomycin biosynthesis. PHS mRNA levels increased only after all the glucose in the medium was exhausted (Martin and Liras, 1989). Also, a phosphatase in neomycin biosynthesis has been shown to be under glucose repression.

Other mechanisms have been proposed and these include decreases in pH and depletion of dissolved O_2. The lowering of pH can come about by the accumulation of undissociated forms of pyruvic and acetic acid due to high glucose concentrations. The regulation of gene expression by pH in *A. nidulans* has been shown to be mediated by a Zn finger TF PacC, which operates with six genes involved in a pH signal transduction pathway; here, full length PacC (73 kDa) is converted to a functional proteolyzed form (29 kDa) under alkaline conditions. Mutation disrupting the *pal A, B, C, F, H* and *I* genes prevent this conversion and results in effects reflecting growth at acidic pH (Negrete-Urtasun *et al.*, 1997). cAMP has been considered to be a possible effector molecule for catabolite repression in some microorganisms where it acts as a positive effector of catabolite repression of inducible catabolic enzymes, since high glucose concentrations inhibit adenylate cyclase (which produces cAMP), thus lowering intracellular concentrations. cAMP binds to a receptor protein to form a complex, which can then interact with promoters for inducible enzymes. It is believed that cAMP is more closely related to phosphate regulation than carbon regulation and, at high levels, may be inhibitory to synthases (Martin and Demain, 1980).

Regulation of secondary metabolism by nitrogen metabolites has been reported in many instances. Ammonium (NH_4^+) is a repressor of enzymes involved in the utilization of other N sources, including nitrite and nitrate reductases, NAD^+-dependent glutamate dehydrogenase, and arginase. In fungi, an enzyme protein appears to be involved in N metabolite regulation and requires NH_4^+, 2-oxoglutarate, and a catalytically active NAD^+ specific glutamate dehydrogenase for repression to occur. Media for antibiotic production frequently utilize slowly metabolized amino acids, resulting in N limitation; for example, the use of proline as the N source in the streptomycin fermentation. The production of patulin by *P. urticae* was delayed by the presence of NH_4^+ in the fermentation medium, especially if added during the seven hour period of de-repression, leading to the formation of secondary enzymes and idiophase proteinases. Secondary enzyme production was more sensitive to this type of regulation than the proteinases (Rollins and Gaucher, 1994). The appearance of the first enzyme of the patulin pathway occurred at an NH_4^+ concentration of 2.5 mM. These authors have also discussed primary nitrogen repression mechanisms in other fungi in explanation of what may have been occurring in *P. urticae*.

The general de-repression of genes regulated by nitrogen (for example, the genes for nitrate or amino acid utilization) operates through the action of a DNA-binding regulatory protein in response to the depletion of the preferred nitrogen source. Control of de-repression is mediated through two signals; a global regulatory gene product, and a pathway-specific gene product. The global regulatory genes are *trans*-acting positive effectors belonging to the GATA family of transcription factors which contain a central GATA core sequence and bind DNA by a cys_2/cys_2-type Zn finger motif. These include AREA (*areA*) from *Aspergillus nidulans*, NIT2 (*nit-2*) from *Neurospora crassa*, and NRE (*nre*) from *P. chrysogenum*, which have a significant degree of sequence identity to each other, especially in the 50 residue region involved in DNA binding.

These proteins may bind other proteins or glutamine (a critical metabolite for N metabolite repression) at the carboxy terminus. The pathway-specific regulatory factors are believed to be activated through binding a specific inducer, and many belong to the GAL4 family of regulatory proteins, which contain a cys_6/Zn_2-type binuclear Zn cluster (unique to fungi) for DNA binding. These proteins interact/cooperate with the global-acting regulators to switch on specific sets of N catabolic genes, depending on substrate availability and N requirements. An example is NIT2, which has been shown to bind to NIT4 (through its Zn finger) to regulate expression of the nitrate reductase (*nit-3*) gene in *N. crassa*. As mentioned above, glutamine is responsible for the specific induction of N de-repression and is believed to operate either unchanged or as a derivative compound, possibly through recognition by a global regulator-pathway specific regulator complex, or by binding individually to the global regulator or the pathway regulator. The nature of the signal transduction pathway which senses repressing levels of glutamine, or whether or not an unidentified factor is involved in glutamine detection (to transfer the signal to the global proteins), are unknown (Marzluf, 1997).

Evans and Ratledge (1985) studied the roles of several enzymes involved with lipid accumulation by the oleaginous yeast *Rhodosporidium toruloides* CBS 14 and noted that AMP deaminase activity was higher in nitrogen-limited cells, irrespective of the N source. The exhaustion of cAMP immediately after N depletion in another lipid accumulating yeast suggested that regulation by the adenylate energy charge could be related to lipogenesis. It was proposed that AMP deaminase could quickly create a low intracellular AMP concentration, leading to a rapid increase of citrate accumulation through inhibition of NAD^+-ICDH. The NH_4^+ produced by the deaminase reaction could then stimulate glycolysis, since it is a positive effector of phosphofructokinase and pyruvate kinase. It also stimulates ATP:citrate lyase activity, which generates acetyl CoA, leading to lipid formation.

Kim *et al.* (1995) showed that the intracellular ammonium level of the citric acid overproducer *A. niger* could be controlled by specific O_2 uptake rate. NH_4^+ has been shown to strongly regulate the glycolytic enzyme phosphofructokinase, and may counteract the feedback inhibition of citrate on this enzyme, thus allowing citrate overproduction to occur. The specific oxygen uptake rate and intracellular NH_4^+ concentration were shown to be closely correlated through pulsed feeding of ammonium to fed-batch cultures.

Production of water-soluble red pigments by *Monascus* sp. was shown to be reliant on ammonium salts as the N source, but appeared to be repressed when NH_4NO_3 was

used as the sole N source in the fermentations. Experiments revealed that, in this case, the NH_4NO_3 was not involved in an N repression process, or in the enhancement of synthase decay, but that the observed effects were due to the poor ability of NH_4NO_3 to donate N to the Schiff-base reaction involved in the transformation of orange precursors to red pigments (Lin and Demain, 1995).

Doull and Vining (1990) showed that N catabolite repression was probably operating in *Streptomyces coelicolor* A3(2) production of actinorhodin, which occurs via a polyketide intermediate and where N-limitation resulted in a higher rate of antibiotic synthesis. Another polyketide-producing member of this genus, *S. ambofaciens*, was also shown to experience N source dependent regulation. In the production of spiramycin, the formation of malonyl CoA, the activated intermediate, is proposed to occur via two pathways. Firstly, oxaloacetate dehydrogenase was shown to be induced (or possibly de-repressed), after growth on NH_4^+ during idiophase; and secondly, acetyl CoA carboxylase was induced after growth on valine during idiophase (Laakel *et al.*, 1994).

N addition to induction/de-repression type mechanisms, other factors may affect how N regulates secondary metabolism. The transport of NH_4^+ will affect its pool size and, therefore, any further interaction with secondary metabolism. In *A. nidulans*, the intracellular concentration of NH_4^+ was shown to regulate the synthesis of some of the protein components of its uptake system by inhibition and repression, whilst in the fungus *Stemphylum botrysum*, the NH_4^+ transport system is regulated by L-amides (Aharonowitz, 1980). In fungi, amino acid transport is also affected by NH_4^+, which inhibits transport due to its uptake into cells. This has been demonstrated with *S. cerevisiae*, in which the NH_4^+ was incorporated into α-amino acids such as glutamate, demonstrating that the enzymic activity of $NADP^+$-glutamate dehydrogenase (anabolic) inhibits amino acid transport, rather than functioning as a regulatory element (Aharonowitz, 1980). Intracellular amino acid pools alter during the growth cycle, and are markedly affected by environmental factors such as NaCl concentration. High amino acid accumulations only occur during stationary, or slow, periods of growth, and it is assumed that the rate of amino acid uptake corresponds with the rate of macromolecular synthesis.

The relationship between aromatic amino acid biosynthesis and secondary metabolism in higher plants has been examined by Jensen *et al.* (1989) and provided another perspective for the regulation of metabolism by N compounds. Aromatic amino acid biosynthesis has been considered to be restricted to chloroplasts. However, evidence has been presented to support the hypothesis than it could also occur in the cytosol, but catalyzed by different isozymes to those involved in the chloroplast pathway. Aromatic amino acid biosynthesis in the cytosol would interface directly with secondary processes occurring here and, therefore, could be regulated or manipulated differently to the chloroplast pathway, although some interaction between the intracellular pools may occur.

Phosphate is another crucial growth-limiting nutrient in many fermentations, and is involved in several different biosynthetic groups, including peptides, polyene macrolides, and tetracyclines. High phosphate concentrations repress the formation of several antibiotics, including *p*-amino benzoic acid synthase (*pabS* gene) in candicidin synthesis. The level of *pabS* mRNA decreased by 95% when high levels of P were added, and this effect was specific to this gene since total mRNA

synthesis was enhanced under these conditions. Subsequently, a P-regulated promoter, upstream of the *pabS* structural gene, was cloned (Martin and Liras, 1989). Studies have been carried out to ascertain if intracellular orthophosphate is the ultimate effector molecule, or if it regulates another intracellular effector to control biosynthetic expression. ATP levels increased after phosphate addition to *Streptomyces griseus*, and this occurred just prior to inhibition of antibiotic synthesis, but the rate of protein production did not change, thus raising the possibility that ATP could act as an intracellular effector.

A variety of mechanisms have been suggested for the control of secondary metabolism by inorganic phosphate (P_i), but they have little experimental support. One possible site for regulation is alkaline phosphatase, whose activity and synthesis is suppressed by P_i. P_i concentrations lower than those found to be needed to support the vegetative growth of plant microbial or animal cells were required for secondary metabolism to occur, since alkaline phosphatase is required for the synthesis of many secondary metabolites and is de-repressed at low P_i concentrations. Phosphatases are often formed prior to the production of antibiotics such as streptomycin, viomycin and vancomycin, and it has been suggested that high concentrations of P_i prevent the synthesis of such antibiotics by repressing these enzymes (Weinberg, 1974). A phosphate-regulated promoter sequence for *pab* genes involved in candicidin synthesis has been determined for *S. griseus*, an organism which has been shown to undergo P_i repression (Doull and Vining, 1990). Also, with respect to phosphate regulation, some biosynthetic intermediates are phosphorylated, whereas the end products are not. In general, microbial phosphatases acting on phosphorylated intermediates are often regulated by feedback inhibition or repression by P_i.

Induction refers to enzymes which are normally absent or barely detectable but which are formed rapidly after the addition of substrate or substrate analogues, and by a process that is the reversal of repression (also a description of de-repression) (Demain, 1968). Thus, it is sometimes difficult to determine whether the stimulatory effect is true induction or a precursor effect. Inducers include those compounds that stimulate biosynthesis when added before idiophase, but not when added during idiophase, when protein synthesis is inhibited by the addition of an inhibitor; they may also be replaced by a non-precursor analogue. By contrast, precursors stimulate synthesis when added during idiophase, even when protein synthesis has been blocked. Initiator accumulation may cause two sequences of events: (1) a pre-existing enzyme, which was only weakly active because of low substrate levels or other competing reactions, now becomes functional, or (2) the initiator induces (or de-represses) the formation of enzymes responsible for product formation. The closure of the usual biosynthetic and energy generating cyclic pathways at the end of tropophase results in an accumulation in the concentrations of initiators, and results in the induction of secondary metabolic pathways (Demain, 1968).

The A-factor (2-*S*-iso-caprolyl-3-*R*-hydroxymethyl-γ-butyrolactone) is a pleiotropic effector responsible for the stimulation of streptomycin production and resistance, and sporulation in *S. griseus* (Martin and Liras, 1989). Its effects are most marked if added at the time of inoculation rather than later (after 48 hours), even with brief treatments of only 3–4 minutes. Although the molecular mechanism of A-factor is unknown, it is reported to have an effect on carbohydrate metabolism, and control occurs at the transcriptional level. Glucose-6-phosphate dehydrogenase (G6PDH)

activity is higher in non-antibiotic-producing mutants and almost undetectable in high-producing mutant strains. After addition of A-factor, G6PDH activity decreases immediately, although it did not affect the isolated pure enzyme, therefore, another compound may be formed in response to A-factor to mediate this effect. One candidate is adenosine diphosphoribose, a breakdown product of $NADP^+$, which can selectively inhibit G6PDH activity and which could lead to the utilization of glucose via alternative routes, resulting in streptomycin production. In *N. crassa*, this compound influenced sporulation, and a similar role in *S. griseus* has been demonstrated (Khokhlov and Tovakova, 1979). A-factor genes have been cloned from various *Streptomyces* spp.; *afsA* from *S. bikiniensis* (A-factor), *afsB* from *S. coelicolor*. The products from these genes are so similar that they may be transferred into A-factor deficient mutants to successfully induce secondary metabolism. It is thought that a cascade of expression is initiated by genes such as *afsB* to control secondary metabolism and differentiation in *Streptomyces* spp. Another gene, *saf* (secondary metabolism activating factor), was cloned from *S. griseus*, and includes a DNA-binding domain in its structure. This acts as a common control factor for at least five extracellular enzymes, pigment formation, and differentiation. It is believed that *saf* may interact directly with regulatory sequences of secondary metabolism, or have an involvement in A-factor formation which, in turn, controls activation of gene clusters for secondary metabolism (Martin and Liras, 1989).

MECHANISMS TO TERMINATE SECONDARY BIOSYNTHESIS

The duration of secondary metabolite biosynthesis ranges from four hours through to several days under non-repressive or non-inhibitory conditions, and also varies between strains and environmental conditions. Feedback regulation has been discussed already, but it also provides the major process for the cessation of biosynthesis. Irreversible decay of one or more of the enzymes of the biosynthetic pathway is another way by which this can be achieved. Natural immobilization of the enzyme complex can occur, as with the soluble bacitracin synthase of *Bacillus licheniformis*, which becomes cell-bound in later growth phase, and this confers protection against intracellular proteases that become active at this time. Gramicidin S synthase is also inactivated by O_2 in a similar process, independent of proteases. Intracellular proteinase activity is responsible for the degradation of the patulin pathway activity in *P. urticae*, and is controlled by the NH_4^+ level in a manner similar to the control of the initiation of biosynthesis (Rollins and Gaucher, 1994). A third means for the cessation of secondary metabolite synthesis is the depletion of intermediary precursors of the final metabolite, but there is no experimental evidence to support this hypothesis (Martin and Demain, 1980).

CONTROL EFFECTS BY ENVIRONMENTAL FACTORS

Inorganic compounds such as phosphate can affect the production of secondary metabolites, even though they are not incorporated into the product. For example, the P_i concentration is usually critical with respect to avoiding excess concentrations. Among the effects observed are increased rates of sugar utilization, mycelial nitrogen content, RNA and DNA and the transient concentration of pyruvate excreted into the

medium. Some pentose phosphate enzymes have been shown to be inhibited by P_i, shifting sugar metabolism to the glycolysis pathway. Manganese (Mn^{2+}) is reported to be a stimulant of secondary metabolite production in *Bacillus*; it cannot be replaced by other elements but is blocked by mRNA and protein synthesis inhibitors, indicating that it could be related to some sort of de-repression control mechanism. Manganese plays a major role in the regulation of the expression of the Mn-peroxidase (MnP) involved in lignin degradation by white rot fungi such as *Phaenerochaete chrysosporium* and *Dichomitus squalens* (Perie and Gold, 1991; Brown *et al.*, 1990). Brown *et al.* (1990) showed that this enzyme was detectable only in cells grown in the presence of Mn^{2+} and that its induction was blocked in the presence of transcriptional inhibitors, suggesting a role for Mn^{2+} as a transcriptional regulator as well as a substrate. The regulation of MnP and lignin peroxidase is believed to be mediated by cAMP since changes in intracellular levels of this compound resulted in the differential regulation of these two enzymes (Boominathan and Reddy, 1992). The lack of LiP activity in cultures with high concentrations of Mn^{2+} could be due to increased levels of phosphodiesterase, which degrades cAMP and is stimulated by Mn^{2+}.

Scott *et al.* (1984) showed that in *P. urticae* Mn was required to induce the patulin biosynthetic pathway beyond the first committed metabolite 6-MSA. In Mn-supplemented cultures, patulin production prevented the accumulation of 6-MSA, as compared to Mn-deficient cultures in which there was minimal production of patulin (Scott *et al.*, 1986a,b). The activity of *m*-hydroxybenzyl alcohol dehydrogenase (a later patulin pathway enzyme) was significantly lower in Mn^{2+} deficient cultures, and a dose-response relationship was established between patulin production and Mn^{2+} concentration.

As discussed earlier, O_2 transfer is another environmental factor which can have critical effects on cell metabolism and is commonly supplied in many fermentations. In some situations, an oversupply of O_2 can be inhibitory to product formation and, therefore, may be involved in regulation of metabolism in some way. O_2 is involved in glucose catabolite repression (which can be overcome by anaerobic shock), and is responsible for diverting glucose metabolism via the pentose phosphate pathway and away from glycolysis. The production of many secondary metabolites is affected by O_2; for example, geosmin is a sesquiterpenoid produced by *P. expansum*, which is stimulated by an O_2–enriched atmosphere (Dionigi and Ingram, 1994). By contrast, red pigment production by *Monascus purpureus* was inhibited under increased aeration conditions, probably because its production requires ethanol as a carbon source and which is favoured under the fermentative growth provided by low O_2.

Lipid addition has often proven beneficial to fermentations by its inhibitory effect of the CoA thioesters of fatty acids on the key enzymes of lipid synthesis, TCA cycle and the HMP pathway (acetyl CoA carboxylase, citrate synthase, and glucose-6-phosphate dehydrogenase), thus preventing diversion into undesirable pathways. Lipids have also been used as the major carbon source, replacing carbohydrates in fermentations that do not utilize polyketide biosyntheses, and which would be feedback-inhibited under these conditions.

Temperature has also been shown to play a role in affecting fermentation processes, with different temperature optima being required for tropophase and idiophase. The optimum temperature for idiophase tends to be below that for normal growth; for example, citric acid production by *A. niger* was optimal at 26.5°C, despite the

optimum for growth being 37°C. Basic control mechanisms can be sensitive to temperature manipulation since the end-product inhibition of the initial enzymes of the amino acid pathways occurs at lower temperatures than those for normal growth. Therefore, the lowering of a culture's temperature after tropophase leads to a greater shut-down of the normal pathways, and thus encouraging the transfer to idiophase metabolism (Demain, 1968).

In studies with Basidiomycetes, secondary metabolite production has been induced by toxic secondary metabolites from other fungi of the same class, leading to retardation of growth and extensive stimulation of synthesis. The process was unrelated to the presence of cell wall materials, as in fungal/plant interactions, and occurred before any cell contact had taken place (Sonnenbichler *et al.*, 1994). For example, fomanosin and fomanoxin from *Heterobasidion annosum* were response-inducing metabolites.

In many industrial fermentation processes, it is desirable to overcome some of these mechanisms in order to achieve overproduction of the desired metabolite. This may be achieved by adding inducers, removal of repressors, and the genetic manipulation of the enzyme-forming systems to reduce the effect of regulation.

Mutation is one means by which this goal can be achieved, with the aim of producing mutants resistant to catabolite repression and constitutive mutants which are able to produce the desired enzyme in the presence of a repressor, or which are able to form enzymes in the absence of an inducer. The reversion technique can produce mutants that often have higher production capabilities than the wild-type grandparent. Reverse auxotrophic mutations, or reversion, can be used to excrete end products of de-repressed enzyme systems or de-sensitized enzymes. Reversions in structural genes can result in enzymes which are structurally altered from the original enzyme and are still active, but differ from the original because they are insensitive to feedback regulation (Demain, 1966). Reversion can affect many genetic characteristics of the culture other than the intended locus, resulting in pleiotropic mutants from the reverse mutation, which are useful for selection (Demain, 1968).

Breaking feedback regulation can be achieved in two ways. The first, and simplest, is to decrease the concentration of the end product; an example of this is in the ornithine fermentation. Ornithine is an intermediate on the unbranched arginine pathway so, by using a mutant of *Corynebacterium glutamicum* deficient in the enzyme catalyzing the step after ornithine, and also feeding low levels of the end-product arginine to overcome inhibition of an earlier enzyme on the pathway, high levels of ornithine are excreted into the culture medium (Demain, 1966, 1968).

If the end product of a branched pathway is the product of interest, the removal of feedback regulation is easier since the reversal of feedback inhibition can be overcome by the other end products of the same pathway. For example, lysine is produced from a pathway that also yields threonine, and these branch after the second step of the pathway. Removal of the enzyme on the threonine branch in a *C. glutamicum* mutant regulated the intracellular concentration of threonine, which inhibited the first enzyme of the pathway. In lysine producing mutants, a mixture of lysine and threonine inhibits this first step by multivalent (or concerted) feedback inhibition. Consequently, low threonine concentrations cause a high level of lysine production.

A second way to overcome feedback regulation is by alteration of enzymes, or

enzyme-forming mechanisms. This is relevant to unbranched pathways where limiting the concentration of an end product is not desirable and requires modification of an enzyme so that it is less sensitive to end-product inhibition or alteration of the enzyme-forming system so it is less prone to end-product inhibition. One way to achieve this is to isolate mutants resistant to growth inhibitory analogues of the desired end product. Here the analogue mimics the natural compound in its feedback function but, since the analogue is unable to be used for macromolecular synthesis, growth is inhibited. In this way, the inhibitory analogue selects for rare mutants with altered enzyme or enzyme-forming systems that are insensitive to feedback regulation and therefore can overproduce and excrete the end product. For example, trifluoroleucine-resistant mutants of *Salmonella typhimurium* excreted leucine as a result of a mutation controlling repression of the leucine pathway (Demain, 1966, 1968).

Conclusions

From the above discussion, it may be seen that plants and microorganisms in general, but the fungi in particular, have evolved sophisticated means to regulate and control the production of secondary metabolites and the pathways of secondary metabolism. What were once mere objects of natural product chemists' curiosity now often play key roles in our daily life and the world's fine chemical industry. Thus, a better understanding of how these processes may be controlled can lead to improved, and perhaps novel, fermentation processes. Nevertheless, it is also clear that we still have but a poor understanding of the basic biochemical mechanisms controlling the effects of O_2 levels and culture conditions on fungal growth and metabolism. Like the early explorer's maps, parts of the coast are filled in; others are mere dotted lines.

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PART 4

Plant Biotechnology