

CHAPTER 4

Media for Industrial Fermentations

INTRODUCTION

DETAILED investigation is needed to establish the most suitable medium for an individual fermentation process, but certain basic requirements must be met by any such medium. All micro-organisms require water, sources of energy, carbon, nitrogen, mineral elements and possibly vitamins plus oxygen if aerobic. On a small scale it is relatively simple to devise a medium containing pure compounds, but the resulting medium, although supporting satisfactory growth, may be unsuitable for use in a large scale process.

On a large scale one must normally use sources of nutrients to create a medium which will meet as many as possible of the following criteria:

1. It will produce the maximum yield of product or biomass per gram of substrate used.
2. It will produce the maximum concentration of product or biomass.
3. It will permit the maximum rate of product formation.
4. There will be the minimum yield of undesired products.
5. It will be of a consistent quality and be readily available throughout the year.
6. It will cause minimal problems during media making and sterilization.
7. It will cause minimal problems in other aspects of the production process particularly aeration and agitation, extraction, purification and waste treatment.

The use of cane molasses, beet molasses, cereal grains, starch, glucose, sucrose and lactose as carbon sources, and ammonium salts, urea, nitrates, corn steep liquor, soya bean meal, slaughter-house waste and fer-

mentation residues as nitrogen sources, have tended to meet most of the above criteria for production media because they are cheap substrates. However, other more expensive pure substrates may be chosen if the overall cost of the complete process can be reduced because it is possible to use simpler procedures. Other criteria are used to select suitable sporulation and inoculation media and these are considered in Chapter 6.

It must be remembered that the medium selected will affect the design of fermenter to be used. For example, the decision to use methanol and ammonia in the single cell protein process developed by ICI plc necessitated the design of a novel fermenter design (MacLennan *et al.*, 1973; Sharp, 1989). The microbial oxidation of hydrocarbons is a highly aerobic and exothermic process. Thus, the production fermenter had to have a very high oxygen transfer capacity coupled with excellent cooling facilities. ICI plc solved these problems by developing an air lift fermenter (see Chapter 7). Equally, if a fermenter is already available this will obviously influence the composition of the medium. Rhodes *et al.* (1955) observed that the optimum concentrations of available nitrogen for griseofulvin production showed some variation with the type of fermenter used. Some aspects of this topic are considered in Chapter 7.

The problem of developing a process from the laboratory to the pilot scale, and subsequently to the industrial scale, must also be considered. A laboratory medium may not be ideal in a large fermenter with a low gas-transfer pattern. A medium with a high viscosity will also need a higher power input for effective stirring. Besides meeting requirements for growth and product formation, the medium may also influence pH variation, foam formation, the oxidation-reduction potential, and the morphological form of the organism. It may also be necessary to provide precursors or

metabolic inhibitors. The medium will also affect product recovery and effluent treatment.

Historically, undefined complex natural materials have been used in fermentation processes because they are much cheaper than pure substrates. However, there is often considerable batch variation because of variable concentrations of the component parts and impurities in natural materials which cause unpredictable biomass and/or product yields. As a consequence of these variations in composition small yield improvements are difficult to detect. Undefined media often make product recovery and effluent treatment more problematical because not all the components of a complex nutrient source will be consumed by the organism. The residual components may interfere with recovery (Chapter 10) and contribute to the BOD of the effluent (Chapter 11).

Thus, although manufacturers have been reluctant to use defined media components because they are more expensive, pure substrates give more predictable yields from batch to batch and recovery, purification and effluent treatment are much simpler and therefore cheaper. Process improvements are also easier to detect when pure substrates are used.

Collins (1990) has given an excellent example of a process producing recombinant protein from *S. cerevisiae* instead of just biomass. The range of growth conditions which can be used is restricted because of factors affecting the stability of the recombinant protein. The control of pH and foam during growth in a fermenter were identified as two important parameters. Molasses would normally be used as the cheapest carbohydrate to grow yeast biomass in a large scale process. However, this is not acceptable for the recombinant protein production because of the difficulties, and incurred costs caused in subsequent purification which result from using crude undefined media components. Collins and co-workers therefore used a defined medium with glucose, sucrose or another suitable carbon source of reasonable purity plus minimal salts, trace elements, pure vitamins and ammonia as the main nitrogen source and for pH control. Other impurities in molasses might have helped to stabilize foams and led to the need to use antifoams.

Aspects of microbial media have also been reviewed by Suomalainen and Oura (1971), Martin and Demain (1978), Iwai and Omura (1982), DeTilly *et al.* (1983), Kuenzi and Auden (1983), Miller and Churchill (1986), Smith (1986) and Priest and Sharp (1989).

Media for culture of animal cells will be discussed later in this chapter.

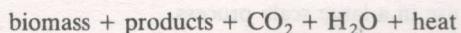
TYPICAL MEDIA

Table 4.1 gives the recipes for some typical media for submerged culture fermentations. These examples are used to illustrate the range of media in use, but are not necessarily the best media in current use.

MEDIUM FORMULATION

Medium formulation is an essential stage in the design of successful laboratory experiments, pilot-scale development and manufacturing processes. The constituents of a medium must satisfy the elemental requirements for cell biomass and metabolite production and there must be an adequate supply of energy for biosynthesis and cell maintenance. The first step to consider is an equation based on the stoichiometry for growth and product formation. Thus for an aerobic fermentation:

carbon + nitrogen + O₂ + other →
and source require-
energy ments
source



This equation should be expressed in quantitative terms, which is important in the economical design of media if component wastage is to be minimal. Thus, it should be possible to calculate the minimal quantities of nutrients which will be needed to produce a specific amount of biomass. Knowing that a certain amount of biomass is necessary to produce a defined amount of product, it should be possible to calculate substrate concentrations necessary to produce required product yields. There may be medium components which are needed for product formation which are not required for biomass production. Unfortunately, it is not always easy to quantify all the factors very precisely.

A knowledge of the elemental composition of a process micro-organism is required for the solution of the elemental balance equation. This information may not be available so that data which is given in Table 4.2 will serve as a guide to the absolute minimum quantities of N, S, P, Mg and K to include in an initial medium recipe. Trace elements (Fe, Zn, Cu, Mn, Co, Mo, B) may also be needed in smaller quantities. An analysis of relative concentrations of individual elements in bacterial cells and commonly used cultivation

| |
|--------------------------------------|
| Itaconic acid (1) |
| Cane molasses |
| ZnSO ₄ |
| ZnSO ₄ ·7H ₂ O |
| CuSO ₄ ·5H ₂ O |
| Amylase (Unde- |
| Ground soybean |
| Autolysed Brew- |
| fractions |
| Distillers dried |
| NZ-amino (enzy- |
| hydrolysate) |
| Lactose |
| MgSO ₄ ·7H ₂ O |
| Hodag KG-1 am- |
| Avermectin (Sta- |
| Cerelose |
| Peptonized milk |
| Autolysed yeast |
| Polyglycol P-200 |
| Distilled water |
| pH |

| |
|--|
| Endotoxin from |
| Molasses |
| Soy flour |
| KH ₂ PO ₄ |
| KH ₂ PO ₄ |
| MgSO ₄ ·7H ₂ O |
| MnSO ₄ ·4H ₂ O |
| FeSO ₄ ·7H ₂ O |
| CaCl ₂ |
| Na(NH ₄) ₂ PO ₄ ·4H ₂ O |
| Lysine (Nakayam) |
| Cane blackstrap |
| Soybean meal hy- |
| (as weight of m- |
| with 6N H ₂ SO ₄ |
| with ammonia |
| CaCO ₃ or MgSO ₄ |
| buffer medium |
| Antifoam agent |

Note. The choice

TABLE 4.1. Some examples of fermentation media

| | | | |
|--|-------------------------|---|----------------------------------|
| Itaconic acid (Nubel and Ratajak, 1962) | | Clavulanic acid (Box, 1980) | |
| Cane molasses (as sugar) | 150 g dm ⁻³ | Glycerol | 1% |
| ZnSO ₄ | 1.0 g dm ⁻³ | Soybean flour | 1.5% |
| ZnSO ₄ ·7H ₂ O | 3.0 g dm ⁻³ | KH ₂ PO ₄ | 0.1% |
| CaSO ₄ ·5H ₂ O | 0.01 g gm ⁻³ | 10% Pluronic L81 antifoam in soya bean oil | 0.2%(v/v) |
| Amylase (Underkofer, 1966) | | Oxytetracycline (Anonymous, 1980) | |
| Ground soybean meal | 1.85% | Starch | 12% + 4% (Additional feeding) |
| Autolyzed Brewers yeast fractions | 1.50% | Technical amylase | 0.1% |
| Distillers dried solubles | 0.76% | Yeast (dry wt.) | 1.5% |
| NZ-amine (enzymatic casein hydrolysate) | 0.65% | CaCO ₃ | 2% |
| Lactose | 4.75% | Ammonium sulphate | 1.5% |
| MgSO ₄ ·7H ₂ O | 0.04% | Lactic acid | 0.13% |
| Hodag KG-1 antifoam | 0.05% | Lard oil | 2% |
| Avermectin (Stapley and Woodruff, 1982) | | Total inorganic salts | 0.01% |
| Cerelose | 45 g | Giberellic acid (Calam and Nixon, 1960) | |
| Peptonized milk | 24 g | Glucose monohydrate | 20 g dm ⁻³ |
| Autolyzed yeast | 2.5 g | MgSO ₄ | 1 g dm ⁻³ |
| Polyglycol P-2000 | 2.5 cm ³ | NH ₄ H ₂ PO ₄ | 2 g dm ⁻³ |
| Distilled water | 1 dm ³ | KH ₂ PO ₄ | 5 g dm ⁻⁴ |
| pH | 7.0 | FeSO ₄ ·7H ₂ O | 0.01 g dm ⁻³ |
| Endotoxin from <i>Bacillus thuringiensis</i> (Holmberg <i>et al.</i> , 1980) | | MnSO ₄ ·4H ₂ O | 0.01 g dm ⁻³ |
| Molasses | 0-4% | ZnSO ₄ ·7H ₂ O | 0.01 g dm ⁻³ |
| Soy flour | 2-6% | CuSO ₄ ·5H ₂ O | 0.01 g dm ⁻³ |
| KH ₂ PO ₄ | 0.5% | Corn steep liquor (as dry solids) | 7.5 g dm ⁻³ |
| KH ₂ PO ₄ | 0.5% | Glutamic acid (Gore <i>et al.</i> , 1968) | |
| MgSO ₄ ·7H ₂ O | 0.005% | Dextrose | 270 g dm ⁻³ |
| MnSO ₄ ·4H ₂ O | 0.003% | NH ₄ H ₂ PO ₄ | 2 g dm ⁻³ |
| FeSO ₄ ·7H ₂ O | 0.001% | (NH ₄) ₂ HPO ₄ | 2 g dm ⁻³ |
| CaCl ₂ | 0.005% | K ₂ SO ₄ | 2 g dm ⁻³ |
| Na(NH ₄) ₂ PO ₄ ·4H ₂ O | 0.15% | MgSO ₄ ·7H ₂ O | 0.5 g dm ⁻³ |
| Lysine (Nakayama, 1972a) | | MnSO ₄ ·4H ₂ O | 0.04 g dm ⁻³ |
| Cane blackstrap molasses | 20% | FeSO ₄ ·7H ₂ O | 0.02 g dm ⁻³ |
| Soybean meal hydrolysate (as weight of meal before hydrolysis with 6N H ₂ SO ₄ and neutralized with ammonia water) | 1.8% | Polyglycol 2000 | 0.3 g dm ⁻³ |
| CaCO ₃ or MgSO ₄ added to buffer medium | | Biotin | 12 µg dm ⁻³ |
| Antifoam agent | | Penicillin | 11 µg dm ⁻³ |
| | | Penicillin (Perlman, 1970) | |
| | | Glucose or molasses (by continuous feed) | 10% of total |
| | | Corn-steep liquor | 4-5% of total |
| | | Phenylacetic acid (by continuous feed) | 0.5-0.8% of total |
| | | Lard oil (or vegetable oil) antifoam by continuous addition | 0.5% of total |
| | | pH to 6.5 to 7.5 by acid or alkali addition | |

Note. The choice of constituents in the ten media is not a haphazard one. The rationale for medium design will be detailed in the remainder of the chapter

TABLE 4.2. Element composition of bacteria, yeasts and fungi (% by dry weight)

| Element | Bacteria (Luria, 1960; Herbert, 1976; Aiba <i>et al.</i> , 1973) | Yeasts (Aiba <i>et al.</i> , 1973; Herbert, 1976) | Fungi (Lilly, 1965; Aiba <i>et al.</i> , 1973) |
|------------|---|---|--|
| Carbon | 50-53 | 45-50 | 40-63 |
| Hydrogen | 7 | 7 | |
| Nitrogen | 12-15 | 7.5-11 | 7-10 |
| Phosphorus | 2.0-3.0 | 0.8-2.6 | 0.4-4.5 |
| Sulphur | 0.2-1.0 | 0.01-0.24 | 0.1-0.5 |
| Potassium | 1.0-4.5 | 1.0-4.0 | 0.2-2.5 |
| Sodium | 0.5-1.0 | 0.01-0.1 | 0.02-0.5 |
| Calcium | 0.01-1.1 | 0.1-0.3 | 0.1-1.4 |
| Magnesium | 0.1-0.5 | 0.1-0.5 | 0.1-0.5 |
| Chloride | 0.5 | — | — |
| Iron | 0.02-0.2 | 0.01-0.5 | 0.1-0.2 |

media quoted by Cooney (1981) showed that some nutrients are frequently added in substantial excess of that required, e.g. P, K; however, others are often near limiting values, e.g. Zn, Cu. The concentration of P is deliberately raised in many media to increase the buffering capacity. These points emphasize the need for considerable attention to be given to medium design.

Some micro-organisms cannot synthesize specific nutrients, e.g. amino acids, vitamins or nucleotides. Once a specific growth factor has been identified it can be incorporated into a medium in adequate amounts as a pure compound or as a component of a complex mixture.

The carbon substrate has a dual role in biosynthesis and energy generation. The carbon requirement for biomass production under aerobic conditions may be estimated from the cellular yield coefficient (*Y*) which is defined as:

$$\frac{\text{Quantity of cell dry matter produced}}{\text{Quantity of carbon substrate utilized}}$$

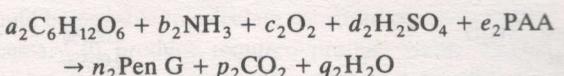
Some values are given in Table 4.3. Thus for bacteria

TABLE 4.3. Cellular yield coefficients (*Y*) of bacteria on different carbon substrates (data from Abbott and Clamen, 1973)

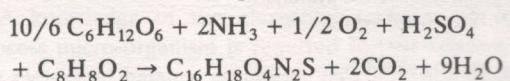
| Substrate | Cellular yield coefficient (g biomass dry wt. g ⁻¹ substrate) |
|--------------------|--|
| Methane | 0.62 |
| n-Alkanes | 1.03 |
| Methanol | 0.40 |
| Ethanol | 0.68 |
| Acetate | 0.34 |
| Malate | 0.36 |
| Glucose (molasses) | 0.51 |

with a *Y* for glucose of 0.5, which is 0.5 g cells g⁻¹ glucose, the concentration of glucose needed to obtain 30 g dm⁻³ cells will be $30/0.5 = 60$ g dm⁻³ glucose. One litre of this medium would also need to contain approximately 3.0 g N, 1.0 g P, 1.0 g K, 0.3 g S and 0.1 g Mg. More details of *Y* values for different micro-organisms and substrates are given by Atkinson and Mavituna (1991b).

An adequate supply of the carbon source is also essential for a product-forming fermentation process. In a critical study, analyses are made to determine how the observed conversion of the carbon source to the product compares with the theoretical maximum yield. This may be difficult because of limited knowledge of the biosynthetic pathways. Cooney (1979) has calculated theoretical yields for penicillin G biosynthesis on the basis of material and energy balances using a biosynthetic pathway based on reaction stoichiometry. The stoichiometry equation for the overall synthesis is:



where a_2 , b_2 , c_2 , d_2 , e_2 , n_2 , p_2 and q_2 are the stoichiometric coefficients and PAA is phenylacetic acid. Solution of this equation yields:



In this instance it was calculated that the theoretical yield was 1.1 g penicillin G g⁻¹ glucose (1837 units mg⁻¹).

Using a simple model for a batch-culture penicillin fermentation it was estimated that 28, 61 and 11% of the glucose consumed was used for cell mass, maintenance and penicillin respectively. When experimental

results of a fed-batch penicillin fermentation were analysed, 26% of the glucose has been used for growth, 70% for maintenance and 6% for penicillin. The maximum experimental conversion yield for penicillin was calculated to be 0.053 g g^{-1} glucose (88.5 units mg^{-1}). Thus, the theoretical conversion value is many times higher than the experimental value. Hersbach *et al.* (1984) concluded that there were six possible biosynthetic pathways for penicillin production and two possible mechanisms for ATP production from NADH and FADH_2 . They calculated that conversion yields by different pathways varied from 638 to 1544 units of penicillin per mg glucose. At that time the best quoted yields were 200 units penicillin per mg glucose. This gives a production of 13 to 29% of the maximum theoretical yield.

The other major nutrient which will be required is oxygen which is provided by aerating the culture, and this aspect is considered in detail in Chapter 9. The design of a medium will influence the oxygen demand of a culture in that the more reduced carbon sources will result in a higher oxygen demand. The amount of oxygen required may be determined stoichiometrically, and this aspect is also considered in Chapter 9. Optimization is dealt with later in this chapter.

WATER

Water is the major component of all fermentation media, and is needed in many of the ancillary services such as heating, cooling, cleaning and rinsing. Clean water of consistent composition is therefore required in large quantities from reliable permanent sources. When assessing the suitability of a water supply it is important to consider pH, dissolved salts and effluent contamination.

The mineral content of the water is very important in brewing, and most critical in the mashing process, and historically influenced the siting of breweries and the types of beer produced. Hard waters containing high CaSO_4 concentrations are better for the English Burton bitter beers and Pilsen type lagers, while waters with a high carbonate content are better for the darker beers such as stouts. Nowadays, the water may be treated by deionization or other techniques and salts added, or the pH adjusted, to favour different beers so that breweries are not so dependent on the local water source. Detailed information is given by Hough *et al.* (1971) and Sentfen (1989).

The reuse or efficient use of water is normally of high priority. When ICI plc and John Brown Engineer-

ing developed a continuous-culture single cell protein (SCP) process at a production scale of 60,000 tonnes per year it was realized that very high costs would be incurred if fresh purified water was used on a once-through basis, since operating at a cell concentration of 30 g biomass (dw) dm^{-3} would require $2700 \times 10^6 \text{ dm}^3$ of water per annum (Ashley and Rodgers, 1986; Sharp, 1989). Laboratory tests to simulate the process showed that the *Methylophilus methylotrophus* could be grown successfully with 86% continuous recycling of supernatant with additions to make up depleted nutrients. This approach was therefore adopted in the full scale process to reduce capital and operating costs and it was estimated that water used on a once through basis without any recycling would have increased water costs by 50% and effluent treatment costs 10-fold.

Water re-use has also been discussed by Topiwala and Khosrovi (1978), Hamer (1979) and Levi *et al.* (1979).

ENERGY SOURCES

Energy for growth comes from either the oxidation of medium components or from light. Most industrial micro-organisms are chemo-organotrophs, therefore the commonest source of energy will be the carbon source such as carbohydrates, lipids and proteins. Some micro-organisms can also use hydrocarbons or methanol as carbon and energy sources.

CARBON SOURCES

Factors influencing the choice of carbon source

It is now recognized that the rate at which the carbon source is metabolized can often influence the formation of biomass or production of primary or secondary metabolites. Fast growth due to high concentrations of rapidly metabolized sugars is often associated with low productivity of secondary metabolites. This has been demonstrated for a number of processes (Table 4.4). At one time the problem was overcome by using the less readily metabolized sugars such as lactose (Johnson, 1952), but many processes now use semi-continuous or continuous feed of glucose or sucrose, discussed in Chapter 2, and later in this chapter (Table 4.15). Alternatively, carbon catabolite regulation might be overcome by genetic modification of the producer organism (Chapter 3).

TABLE 4.4. Carbon catabolite regulation of metabolite biosynthesis

| Metabolite | Micro-organism | Interfering carbon source | Reference |
|--|----------------------------------|---------------------------|---|
| Griseofulvin | <i>Penicillium griseofulvin</i> | Glucose | Rhodes (1963); Rhodes <i>et al.</i> (1955) |
| Penicillin Cephalosporin | <i>P. chrysogenum</i> | Glucose | Pirt and Rhigelato (1967) |
| | <i>Cephalosporium acremonium</i> | Glucose | Matsumura <i>et al.</i> (1978) |
| Aurantin | <i>Bacillus aurantinus</i> | Glycerol | Nishikiori <i>et al.</i> (1978) |
| α -Amylase | <i>B. licheniformis</i> | Glucose | Priest and Sharp (1989) |
| Bacitracin | <i>B. licheniformis</i> | Glucose | Weinberg (1967) |
| Puromycin | <i>Streptomyces alboniger</i> | Glucose | Sankaran and Pogell (1975) |
| Actinomycin Cephamycin C | <i>S. antibioticus</i> | Glucose | Marshall <i>et al.</i> (1968) |
| | <i>S. clavuligerus</i> | Glycerol | Aharonowitz and Demain (1978) |
| Neomycin | <i>S. fradiae</i> | Glucose | Majumdar and Majumdar (1965) |
| Cycloserine Streptomycin Kanamycin | <i>S. graphalus</i> | Glycerol | Svensson <i>et al.</i> (1983) |
| | <i>S. griseus</i> | Glucose | Inamine <i>et al.</i> (1969) |
| | <i>S. kanamyceticus</i> | Glucose | Basek and Majumdar (1973) |
| Novobiocin | <i>S. niveus</i> | Citrate | Kominek (1972) |
| Siomycin | <i>S. sioyaensis</i> | Glucose | Kimura (1967) |

The main product of a fermentation process will often determine the choice of carbon source, particularly if the product results from the direct dissimilation of it. In fermentations such as ethanol or single-cell protein production where raw materials are 60 to 77% of the production cost, the selling price of the product will be determined largely by the cost of the carbon source (Whitaker, 1973; Moo-Young, 1977). It is often part of a company development programme to test a range of alternative carbon sources to determine the yield of product and its influence on the process and the cost of producing biomass and/or metabolite. This enables a company to use alternative substrates, depending on price and availability in different locations, and remain competitive. Up to ten different carbon sources have been or are being used by Pfizer Ltd for an antibiotic production process depending on the geographical location of the production site and prevailing economics (Stowell, 1987).

The purity of the carbon source may also affect the choice of substrate. For example, metallic ions must be removed from carbohydrate sources used in some citric acid processes (Karrow and Waksman, 1947; Woodward *et al.*, 1949; Smith *et al.*, 1974).

The method of media preparation, particularly sterilization, may affect the suitability of carbohydrates for individual fermentation processes. It is often best to sterilize sugars separately because they may react with ammonium ions and amino acids to form black nitro-

gen containing compounds which will partially inhibit the growth of many micro-organisms. Starch suffers from the handicap that when heated in the sterilization process it gelatinizes, giving rise to very viscous liquids, so that only concentrations of up to 2% can be used without modification (Solomons, 1969).

The choice of substrate may also be influenced by government legislation. Within the European Economic Community (EEC), the use of beet sugar and molasses is encouraged, and the minimum price controlled. The quantity of imported cane sugar and molasses is carefully monitored and their imported prices set so that they will not be competitive with beet sugar. If the world market sugar price is very low then the EEC fermentation industry will be at a disadvantage unless it receives realistic subsidies (Coombs, 1987). Refunds for a defined list of products are available in the EEC when sugar and starch are used as substrates. Legislation for recognition of new products is time consuming and manufacturers may be uncertain as to whether they would benefit from carbon substrate refunds. This uncertainty has meant that some manufacturers might prefer to site factories for new products outside the EEC (Gray, 1987).

Local laws may also dictate the substrates which may be used to make a number of beverages. In the Isle of Man, the Manx Brewers Act (1874) forbids the use of ingredients other than malt, sugar and hops in the brewing of beer. There are similar laws applying to

beer products to be made on the Isle of Man, only if the geographical location

CARBOHYDRATES

It is common for carbon sources to be most widely used from natural materials, potato starches can be directly used in Starch may be used and enzymes (solids and as a major component in Japan for hydrolysis to make them available.

Barley grain treated to contain a Malt is the in many countries from malted

Sucrose is common in impure form which are the solutions in production of ethanol, SC. Microbial gums were used for SC in 1986. Molar production of antibiotics, SC

TABLE 4.5. CARBOHYDRATES

| |
|-----------------|
| Starch |
| Sucrose |
| Reducing sugars |
| Other sugars |
| Hemicellulose |
| Cellulose |

beer production in Germany. Scotch malt whisky may be made only from barley malt, water and yeast. Within France, many wines may be called by a certain name only if the producing vineyard is within a limited geographical locality.

Examples of commonly used carbon sources

CARBOHYDRATES

It is common practice to use carbohydrates as the carbon source in microbial fermentation processes. The most widely available carbohydrate is starch obtained from maize grain. It is also obtained from other cereals, potatoes and cassava. Analysis data for these substrates can be obtained from Atkinson and Mavituna (1991a). Maize and other cereals may also be used directly in a partially ground state, e.g. maize chips. Starch may also be readily hydrolysed by dilute acids and enzymes to give a variety of glucose preparations (solids and syrups). Hydrolysed cassava starch is used as a major carbon source for glutamic acid production in Japan (Minoda, 1986). Syrups produced by acid hydrolysis may also contain toxic products which may make them unsuitable for particular processes.

Barley grains may be partially germinated and heat treated to give the material known as malt, which contains a variety of sugars besides starch (Table 4.5). Malt is the main substrate for brewing beer and lager in many countries. Malt extracts may also be prepared from malted grain.

Sucrose is obtained from sugar cane and sugar beet. It is commonly used in fermentation media in a very impure form as beet or cane molasses (Table 4.6), which are the residues left after crystallization of sugar solutions in sugar refining. Molasses is used in the production of high-volume/low-value products such as ethanol, SCP, organic and amino acids and some microbial gums. In 1980, 300,000 tons of cane molasses were used for amino acid production in Japan (Minoda, 1986). Molasses or sucrose also may be used for production of higher value/low-bulk products such as antibiotics, speciality enzymes, vaccines and fine chemi-

cals (Coombs, 1987). The cost of molasses will be very competitive when compared with pure carbohydrates. However, molasses contains many impurities and molasses-based fermentations will often need a more expensive and complicated extraction/purification stage to remove the impurities and effluent treatment will be more expensive because of the unutilized waste materials which are still present. Some new processes may require critical evaluation before the final decision is made to use molasses as the main carbon substrate.

The use of lactose and crude lactose (milk whey powder) in media formulations is now extremely limited since the introduction of continuous-feeding processes utilizing glucose, discussed in a later section of this chapter.

Corn steep liquor (Table 4.7) is a by-product after starch extraction from maize. Although primarily used as a nitrogen source, it does contain lactic acid, small amounts of reducing sugars and complex polysaccharides. Certain other materials of plant origin, usually included as nitrogen sources, such as soyabean meal and Pharmamedia, contain small but significant amounts of carbohydrate.

OILS AND FATS

Oils were first used as carriers for antifoams in antibiotic processes (Solomons, 1969). Vegetable oils (olive, maize, cotton seed, linseed, soya bean, etc.) may also be used as carbon substrates, particularly for their content of the fatty acids, oleic, linoleic and linolenic acid, because costs are competitive with those of carbohydrates. In an analysis of commodity prices for sugar, soya bean oil and tallow between 1978 and 1985, it would have been cheaper on an available energy basis to use sugar during 1978 to mid 1979 and late 1983 to 1985, whereas oil would have been the chosen substrate in the intervening period (Stowell, 1987).

Bader *et al.* (1984) discussed factors favouring the use of oils instead of carbohydrates. A typical oil contains approximately 2.4 times the energy of glucose on a per weight basis. Oils also have a volume advantage as it would take 1.24 dm³ of soya bean oil to add 10 kcal of energy to a fermenter, whereas it would take 5 dm³ of glucose or sucrose assuming that they are being added as 50% w/w solutions. Ideally, in any fermentation process, the maximum working capacity of a vessel should be used. Oil based fed-batch fermentations permit this procedure to operate more successfully than those using carbohydrate feeds where a larger spare capacity must be catered for to allow for responses to a sudden reduction in the residual nutrient level (Stowell, 1987). Oils also have antifoam properties

TABLE 4.5. Carbohydrate composition of barley malt (Harris, 1962) (expressed as % dry weight of total)

| | |
|-----------------|-------|
| Starch | 58-60 |
| Sucrose | 3-5 |
| Reducing sugars | 3-4 |
| Other sugars | 2 |
| Hemicellulose | 6-8 |
| Cellulose | 5 |

TABLE 4.6. Analysis of beet and cane molasses (Rhodes and Fletcher, 1966) (expressed as % of total w/v)

| | Beet | Cane |
|--------------|------|------|
| Sucrose | 48.5 | 33.4 |
| Raffinose | 1.0 | 0 |
| Invert sugar | 1.0 | 21.2 |

Remainder is non-sugar.

which may make downstream processing simpler, but normally they are not used solely for this purpose.

Stowell (1987) reported the results of a Pfizer antibiotic process operated with a range of oils and fats on a laboratory scale. On a purely technical basis glycerol trioleate was the most suitable substrate. In the UK however, when both technical and economic factors are considered, soyabean oil or rapeseed oil are the preferred substrates. Glycerol trioleate is known to be used in some fermentations where substrate purity is an important consideration. Methyl oleate has been used as the sole carbon substrate in cephalosporin production (Pan *et al.*, 1982).

HYDROCARBONS AND THEIR DERIVATIVES

There has been considerable interest in hydrocarbons. Development work has been done using n-alkanes for production of organic acids, amino acids, vitamins and co-factors, nucleic acids, antibiotics, enzymes and proteins (Fukui and Tanaka, 1980). Methane, methanol and n-alkanes have all been used as substrates for biomass production (Hamer, 1979; Levi *et al.*, 1979; Drozd, 1987; Sharp, 1989).

Drozd (1987) discussed the advantages and disadvantages of hydrocarbons and their derivatives as fermentation substrates, particularly with reference to cost, process aspects and purity. In processes where the feedstock costs are an appreciable fraction of the total manufacturing cost, cheap carbon sources are important. In the 1960s and early 1970s there was an incentive to consider using oil or natural gas derivatives as carbon substrates as costs were low and sugar prices were high. On a weight basis n-alkanes have approximately twice the carbon and three times the energy content of the same weight of sugar. Although petroleum-type products are initially impure they can be refined to obtain very pure products in bulk quantities which would reduce the amount of effluent treatment and downstream processing. At this time the view was also held that hydrocarbons would not be subject to the same fluctuations in cost as agriculturally derived feedstocks because it would be a stable priced commodity and might be used to provide a substrate

TABLE 4.7. Partial analysis of corn-steep liquor (Belik *et al.*, 1957; Misecka and Zelinka, 1959; Rhodes and Fletcher, 1966)

| | |
|---------------------------------------|--------------------------------|
| Total solids | 51% w/v |
| Acidity as lactic acid | 15% w/v |
| Free reducing sugars | 5.6% w/v |
| Free reducing sugars after hydrolysis | 6.8% w/v |
| Total nitrogen | 4% w/v |
| Amino acids as % of nitrogen | |
| Alanine | 25 |
| Arginine | 8 |
| Glutamic acid | 8 |
| Leucine | 6 |
| Proline | 5 |
| Isoleucine | 3.5 |
| Threonine | 3.5 |
| Valine | 3.5 |
| Phenylalanine | 2.0 |
| Methionine | 1.0 |
| Cystine | 1.0 |
| Ash | 1.25% w/v |
| Potassium | 20% |
| Phosphorus | 1-5% |
| Sodium | 0.3-1% |
| Magnesium | 0.003-0.3% |
| Iron | 0.01-0.3% |
| Copper } | 0.01-0.03% |
| Calcium } | |
| Zinc | 0.003-0.08% |
| Lead | |
| Silver } | 0.001-0.003% |
| Chromium | |
| B Vitamins | |
| Aneurine | 41-49 $\mu\text{g g}^{-1}$ |
| Biotin | 0.34-0.38 $\mu\text{g g}^{-1}$ |
| Calcium pantothenate | 14.5-21.5 $\mu\text{g g}^{-1}$ |
| Folic acid | 0.26-0.6 $\mu\text{g g}^{-1}$ |
| Nicotinamide | 30-40 $\mu\text{g g}^{-1}$ |
| Riboflavin | 3.9-4.7 $\mu\text{g g}^{-1}$ |

Also niacin and pyridoxine

for conversion to microbial protein (SCP) for economic animal and/or human consumption. Sharp (1989) gives a very good account of market considerations of changes in price and how this would affect the price of SCP. The SCP would have had to have been cheaper or as cheap as soya meal to be marketed as an animal feed supplement. It is evident that both ICI plc and Shell plc made very careful assessments of likely future prices of soya meal during process evaluation.

SCP processes were developed by BP plc (Toprina from yeast grown on n-alkanes), ICI plc (Pruteen from bacteria grown on methanol), Hoechst / UBHE (Probion from bacteria on methanol) and Shell plc (bacteria on methane). Only BP plc and ICI plc eventually developed SCP at a production scale as an animal feed

supplement (Sharp, 1989). BP's product was produced by an Italian subsidiary company, but rapidly withdrawn from manufacture because of Italian government opposition and the price of feed stock quadrupling in 1973. At this time the crude oil exporting nations (OPEC) had collectively raised the price of crude oil sold on the world market. In spite of the significant increase in the cost of crude oil and its derivatives, as well as recognizing the importance of competition from soya bean meal, the ICI plc directorate gave approval to build a full scale plant in 1976. Pruteen was marketed in the 1980s but eventually withdrawn because it could not compete with soya bean meal prices as an animal feed supplement.

Drozd (1987) has made a detailed study of hydrocarbon feedstocks and concluded that the cost of hydrocarbons does not make them economically attractive bulk feedstocks for the production of established products or potential new products where feedstock costs are an appreciable fraction of manufacturing costs of low-value bulk products. In SCP production, raw materials account for three quarters of the operating or variable costs and about half of the total costs of manufacture (Sharp, 1989; see also Chapter 12). It was considered that hydrocarbons and their derivatives might have a potential role as feedstocks in the microbial production of higher value products such as intermediates, pharmaceuticals, fine chemicals and agricultural chemicals (Drozd, 1987).

NITROGEN SOURCES

Examples of commonly used nitrogen sources

Most industrially used micro-organisms can utilize inorganic or organic sources of nitrogen. Inorganic nitrogen may be supplied as ammonia gas, ammonium salts or nitrates (Hunter, 1972). Ammonia has been used for pH control and as the major nitrogen source in a defined medium for the commercial production of human serum albumin by *Saccharomyces cerevisiae* (Collins, 1990). Ammonium salts such as ammonium sulphate will usually produce acid conditions as the ammonium ion is utilized and the free acid will be liberated. On the other hand nitrates will normally cause an alkaline drift as they are metabolized. Ammonium nitrate will first cause an acid drift as the ammonium ion is utilized, and nitrate assimilation is repressed. When the ammonium ion has been exhausted, there is an alkaline drift as the nitrate is used as an alternative nitrogen source (Morton and MacMil-

lan, 1954). One exception to this pattern is the metabolism of *Gibberella fujikuroi* (Borrow *et al.*, 1961, 1964). In the presence of nitrate the assimilation of ammonia is inhibited at pH 2.8–3.0. Nitrate assimilation continues until the pH has increased enough to allow the ammonia assimilation mechanism to restart.

Organic nitrogen may be supplied as amino acid, protein or urea. In many instances growth will be faster with a supply of organic nitrogen, and a few micro-organisms have an absolute requirement for amino acids. It might be thought that the main industrial need for pure amino acids would be in the deliberate addition to amino acid requiring mutants used in amino acid production. However, amino acids are more commonly added as complex organic nitrogen sources which are non-homogeneous, cheaper and readily available. In lysine production, methionine and threonine are obtained from soybean hydrolysate since it would be too expensive to use the pure amino acids (Nakayama, 1972a).

Other proteinaceous nitrogen compounds serving as sources of amino acids include corn-steep liquor, soya meal, peanut meal, cotton-seed meal (Pharmamedia, Table 4.8; and Proflo), Distillers' solubles, meal and yeast extract. Analysis of many of these products which include amino acids, vitamins and minerals are given by Miller and Churchill (1986) and Atkinson and Mavutuna (1991a). In storage these products may be affected by moisture, temperature changes and ageing.

Chemically defined amino acid media devoid of protein are necessary in the production of certain vaccines when they are intended for human use.

Factors influencing the choice of nitrogen source

Control mechanisms exist by which nitrate reductase, an enzyme involved in the conversion of nitrate to ammonium ion, is repressed in the presence of ammonia (Brown *et al.*, 1974). For this reason ammonia or ammonium ion is the preferred nitrogen source. In fungi that have been investigated, ammonium ion represses uptake of amino acids by general and specific amino acid permeases (Whitaker, 1976). In *Aspergillus nidulans*, ammonia also regulates the production of alkaline and neutral proteases (Cohen, 1973). Therefore, in mixtures of nitrogen sources, individual nitrogen components may influence metabolic regulation so that there is preferential assimilation of one component until its concentration has diminished.

It has been shown that antibiotic production by many micro-organisms is influenced by the type and

TABLE 4.8. The composition of Pharmamedia (Traders Protein, Southern Cotton Oil Company, Division of Archer Daniels Midland Co.)

| Component | Quantity |
|------------------------------|---------------------------|
| Total solids | 99% |
| Carbohydrate | 24.1% |
| Reducing sugars | 1.2% |
| Non reducing sugars | 1.2% |
| Protein | 57% |
| Amino nitrogen | 4.7% |
| Components of amino nitrogen | |
| Lysine | 4.5% |
| Leucine | 6.1% |
| Isoleucine | 3.3% |
| Threonine | 3.3% |
| Valine | 4.6% |
| Phenylalanine | 5.9% |
| Tryptophan | 1.0% |
| Methionine | 1.5% |
| Cystine | 1.5% |
| Aspartic acid | 9.7% |
| Serine | 4.6% |
| Proline | 3.9% |
| Glycine | 3.8% |
| Alanine | 3.9% |
| Tyrosine | 3.4% |
| Histidine | 3.0% |
| Arginine | 12.3% |
| Mineral components | |
| Calcium | 2530 ppm |
| Chloride | 685 ppm |
| Phosphorus | 13100 ppm |
| Iron | 94 ppm |
| Sulphate | 18000 ppm |
| Magnesium | 7360 ppm |
| Potassium | 17200 ppm |
| Fat | 4.5% |
| Vitamins | |
| Ascorbic acid | 32.0 mg kg ⁻¹ |
| Thiamine | 4.0 mg kg ⁻¹ |
| Riboflavin | 4.8 mg kg ⁻¹ |
| Niacin | 83.3 mg kg ⁻¹ |
| Pantothenic acid | 12.4 mg kg ⁻¹ |
| Choline | 3270 mg kg ⁻¹ |
| Pyridoxine | 16.4 mg kg ⁻¹ |
| Biotin | 1.5 mg kg ⁻¹ |
| Folic acid | 1.6 mg kg ⁻¹ |
| Inositol | 10800 mg kg ⁻¹ |

concentration of the nitrogen source in the culture medium (Aharonowitz, 1980). Antibiotic production may be inhibited by a rapidly utilized nitrogen source (NH_4^+ , NO_3^- , certain amino acids). The antibiotic production only begins to increase in the culture broth after most of the nitrogen source has been consumed.

In shake flask media experiments, salts of weak acids (e.g. ammonium succinate) may be used to serve as a nitrogen source and eradicate the source of a strong acid pH change due to chloride or sulphate ions which would be present if ammonium chloride or sulphate were used as the nitrogen source. This procedure makes it possible to use lower concentrations of phosphate to buffer the medium. High phosphate concentrations inhibit production of many secondary metabolites (see Minerals Section).

The use of complex nitrogen sources for antibiotic production has been common practice. They are thought to help create physiological conditions in the trophophase which favour antibiotic production in the idiophase (Martin and McDaniel, 1977). For example, in the production of polyene antibiotics, soybean meal has been considered a good nitrogen source because of the balance of nutrients, the low phosphorus content and slow hydrolysis. It has been suggested that this gradual breakdown prevents the accumulation of ammonium ions and repressive amino acids. These are probably some of the reasons for the selection of ideal nitrogen sources for some secondary metabolites (Table 4.9.).

In gibberellin production the nitrogen source has been shown to have an influence on directing the production of different gibberellins and the relative proportions of each type (Jefferys, 1970).

Other pre-determined aspects of the process can also influence the choice of nitrogen source. Rhodes (1963) has shown that the optimum concentration of available nitrogen for griseofulvin production showed some variation depending on the form of inoculum and the type of fermenter being used. Obviously these factors must be borne in mind in the interpretation of results in media-development programmes.

Some of the complex nitrogenous material may not be utilized by a micro-organism and create problems in downstream processing and effluent treatment. This can be an important factor in the final choice of substrate.

MINERALS

All micro-organisms require certain mineral elements for growth and metabolism (Hughes and Poole, 1989, 1991). In many media, magnesium, phosphorus, potassium, sulphur, calcium and chlorine are essential components, and because of the concentrations required, they must be added as distinct components. Others such as cobalt, copper, iron, manganese, molyb-

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TABLE 4

* KH_2PO_4
 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
 KCl
 CaCO_3
 $\text{FeSO}_4 \cdot 4\text{H}_2\text{O}$
 $\text{ZnSO}_4 \cdot 8\text{H}_2\text{O}$
 $\text{MnSO}_4 \cdot \text{H}_2\text{O}$
 $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$
 Na_2MoO_4

* Complex
mally con
phate.

TABLE 4.9. Best nitrogen sources for some secondary metabolites

| Product | Main nitrogen source(s) | Reference |
|--------------|---|-------------------------------|
| Penicillin | Corn-steep liquor | Moyer and Coghill (1946) |
| Bacitracin | Peanut granules | Inskeep <i>et al.</i> (1951) |
| Riboflavin | Pancreatic digest of gelatine | Malzahn <i>et al.</i> (1959) |
| Novobiocin | Distillers' solubles | Hoeksema and Smith (1961) |
| Rifomycin | Pharmamedia | Sensi and Thiemann (1967) |
| Gibberellins | Soybean meal, $(\text{NH}_4)_2\text{SO}_4$ | Jefferys (1970) |
| | Ammonium salt and natural plant nitrogen source | |
| Butirosin | Dried beef blood or haemoglobin with $(\text{NH}_4)_2\text{SO}_4$ | Claridge <i>et al.</i> (1974) |
| Polyenes | Soybean meal | Martin and MacDaniel (1977) |

denum and zinc are also essential but are usually present as impurities in other major ingredients. There is obviously a need for batch analysis of media components to ensure that this assumption can be justified, otherwise there may be deficiencies or excesses in different batches of media. See Tables 4.7 and 4.8 for analysis of corn steep liquor and Pharmamedia, and Miller and Churchill (1986) for analysis of other media ingredients of plant and animal origin. When synthetic media are used the minor elements will have to be added deliberately. The form in which the minerals are usually supplied and the concentration ranges are given in Table 4.10. As a consequence of product composition analysis, as outlined earlier in this chapter, it is possible to estimate the amount of a specific mineral for medium design, e.g. sulphur in penicillins and cephalosporins, chlorine in chlortetracycline.

The concentration of phosphate in a medium, particularly laboratory media in shake flasks, is often much higher than that of other mineral components. Part of this phosphate is being used as a buffer to minimize pH

changes when external control of the pH is not being used.

In specific processes the concentration of certain minerals may be very critical. Some secondary metabolic processes have a lower tolerance range to inorganic phosphate than vegetative growth. This phosphate should be sufficiently low as to be assimilated by the end of trophophase. In 1950, Garner *et al.* suggested that an important function of calcium salts in fermentation media was to precipitate excess inorganic phosphates, and suggested that the calcium indirectly improved the yield of streptomycin. The inorganic phosphate concentration also influences production of bacitracins, citric acid (surface culture), ergot, monomycin, novobiocin, oxytetracycline, polyenes, ristomycin, rifamycin Y, streptomycin, vancomycin and viomycin (Sensi and Thieman, 1967; Demain, 1968; Liu *et al.*, 1970; Mertz and Doolin, 1973; Weinberg, 1974). However, pyrrolnitrin (Arima *et al.*, 1965), bicyclomycin (Miyoshi *et al.*, 1972), thiopeptin (Miyairi *et al.*, 1970) and methylenomycin (Hobbs *et al.*, 1992) are produced in a medium containing a high concentration of phosphate. Two monomycin antibiotics are selectively produced by *Streptomyces jamaicensis* when the phosphate is 0.1 mM or 0.4 mM (Hall and Hassall, 1970). Phosphate regulation has also been discussed by Weinberg (1974), Aharonowitz and Demain (1977), Martin and Demain (1980), Iwai and Omura (1982) and Demain and Piret (1991).

In a recent review of antibiotic biosynthesis, Liras *et al.* (1990) recognized target enzymes which were (a) repressed by phosphate, (b) inhibited by phosphate, or (c) repression of an enzyme occurs but phosphate repression is not clearly proved. A phosphate control sequence has also been isolated and characterized from the phosphate regulated promoter that controls biosynthesis of candididin.

Weinberg (1970) has reviewed the nine trace ele-

TABLE 4.10. The range of typical concentrations of mineral components (g dm^{-3})

| Component | Range |
|---|------------------------------------|
| * KH_2PO_4 | 1.0–4.0 (part may be as buffer) |
| $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ | 0.25–3.0 |
| KCl | 0.5–12.0 |
| CaCO_3 | 5.0–17.0 |
| $\text{FeSO}_4 \cdot 4\text{H}_2\text{O}$ | 0.01–0.1 |
| $\text{ZnSO}_4 \cdot 8\text{H}_2\text{O}$ | 0.1–1.0 |
| $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ | 0.01–0.1 |
| $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ | 0.003–0.01 |
| $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ | 0.01–0.1 |

* Complex media derived from plant and animal materials normally contain a considerable concentration of inorganic phosphate.

ments of biological interest (Atomic numbers 23–30, 42). Of these nine, the concentrations of manganese, iron and zinc are the most critical in secondary metabolism. In every secondary metabolic system in which sufficient data has been reported, the yield of the product varies linearly with the logarithmic concentration of the 'key' metal. The linear relationship does not apply at concentrations of the metal which are either insufficient, or toxic, to cell growth. Some of the primary and secondary microbial products whose yields are affected by concentrations of trace metals greater than those required for maximum growth are given in Table 4.11.

Chlorine does not appear to play a nutritional role in the metabolism of fungi (Foster, 1949). It is, however, required by some of the halophilic bacteria (Larsen, 1962). Obviously, in those fermentations where a chlorine-containing metabolite is to be produced the synthesis will have to be directed to ensure that the non-chloro-derivative is not formed. The most important compounds are chlortetracycline and griseofulvin. In griseofulvin production, adequate available chloride is provided by the inclusion of at least 0.1% KCl (Rhodes *et al.*, 1955), as well as the chloride provided by the complex organic materials included as nitrogen sources. Other chlorine containing metabolites are caldriomycin, nornidulin and mollisin.

Chelators

Many media cannot be prepared or autoclaved without the formation of a visible precipitate of insoluble

metal phosphates. Gaunt *et al.* (1984) demonstrated that when the medium of Mandels and Weber (1969) was autoclaved, a white precipitate of metal ions formed, containing all the iron and most of the calcium, manganese and zinc present in the medium.

The problem of insoluble metal phosphate(s) may be eliminated by incorporating low concentrations of chelating agents such as ethylene diamine tetraacetic acid (EDTA), citric acid, polyphosphates, etc., into the medium. These chelating agents preferentially form complexes with the metal ions in a medium. The metal ions then may be gradually utilized by the micro-organism (Hughes and Poole, 1991). Gaunt *et al.* (1984) were able to show that the precipitate was eliminated from Mandel and Weber's medium by the addition of EDTA at 25 mg dm⁻³. It is important to check that a chelating agent does not cause inhibition of growth of the micro-organism which is being cultured.

In many media, particularly those commonly used in large scale processes, there may not be a need to add a chelating agent as complex ingredients such as yeast extracts or proteose peptones will complex with metal ions and ensure gradual release of them during growth (Ramamoorthy and Kushner, 1975).

GROWTH FACTORS

Some micro-organisms cannot synthesize a full complement of cell components and therefore require pre-formed compounds called growth factors. The growth factors most commonly required are vitamins, but there may also be a need for specific amino acids, fatty acids

TABLE 4.11. Trace elements influencing primary and secondary metabolism

| Product | Trace element(s) | Reference |
|-----------------|------------------|--------------------------------|
| Bacitracin | Mn | Weinberg and Tonnis (1966) |
| Protease | Mn | Mizusawa <i>et al.</i> (1966) |
| Gentamicin | Co | Tilley <i>et al.</i> (1975) |
| Riboflavin | Fe, Co | Hickey (1945) |
| | Fe | Tanner <i>et al.</i> (1945) |
| Mitomycin | Fe | Weinberg (1970) |
| Monensin | Fe | Weinberg (1970) |
| Actinomycin | Fe, Zn | Katz <i>et al.</i> (1958) |
| Candidin | Fe, Zn | Weinberg (1970) |
| Chloramphenicol | Fe, Zn | Gallicchio and Gottlieb (1958) |
| Neomycin | Fe, Zn | Majumdar and Majumdar (1965) |
| Patulin | Fe, Zn | Brack (1947) |
| Streptomycin | Fe, Zn | Weinberg (1970) |
| Citric acid | Fe, Zn, Cu | Shu and Johnson (1948) |
| Penicillin | Fe, Zn, Cu | Foster <i>et al.</i> (1943) |
| | | Koffler <i>et al.</i> (1947) |
| Griseofulvin | Zn | Grove (1967) |

or sterols. Many of the natural carbon and nitrogen sources used in media formulations contain all or some of the required growth factors (Atkinson and Mavituna, 1991a). When there is a vitamin deficiency it can often be eliminated by careful blending of materials (Rhodes and Fletcher, 1966). It is important to remember that if only one vitamin is required it may be occasionally more economical to add the pure vitamin, instead of using a larger bulk of a cheaper multiple vitamin source. Calcium pantothenate has been used in one medium formulation for vinegar production (Beaman, 1967). In processes used for the production of glutamic acid, limited concentrations of biotin must be present in the medium (see Chapter 3). Some production strains may also require thiamine (Kinoshita and Tanaka, 1972).

NUTRIENT RECYCLE

The need for water recycling in ICI plc's continuous-culture SCP process has already been discussed in an earlier section of this chapter. It was shown that *M. methylotrophus* could be grown in a medium containing 86% recycled supernatant plus additional fresh nutrients to make up losses. This approach made it possible to reduce the costs of media components, media preparation and storage facilities (Ashley and Rodgers, 1986; Sharp, 1989).

BUFFERS

The control of pH may be extremely important if optimal productivity is to be achieved. A compound may be added to the medium to serve specifically as a buffer, or may also be used as a nutrient source. Many media are buffered at about pH 7.0 by the incorporation of calcium carbonate (as chalk). If the pH decreases the carbonate is decomposed. Obviously, phosphates which are part of many media also play an important role in buffering. However, high phosphate concentrations are critical in the production of many secondary metabolites (see Minerals section earlier in this chapter).

The balanced use of the carbon and nitrogen sources will also form a basis for pH control as buffering capacity can be provided by the proteins, peptides and amino acids, such as in corn-steep liquor. The pH may also be controlled externally by addition of ammonia or sodium hydroxide and sulphuric acid (Chapter 8).

THE ADDITION OF PRECURSORS AND METABOLIC REGULATORS TO MEDIA

Some components of a fermentation medium help to regulate the production of the product rather than support the growth of the micro-organism. Such additives include precursors, inhibitors and inducers, all of which may be used to manipulate the progress of the fermentation.

Precursors

Some chemicals, when added to certain fermentations, are directly incorporated into the desired product. Probably the earliest example is that of improving penicillin yields (Moyer and Coghill, 1946, 1947). A range of different side chains can be incorporated into the penicillin molecule. The significance of the different side chains was first appreciated when it was noted that the addition of corn-steep liquor increased the yield of penicillin from 20 units cm^{-3} to 100 units cm^{-3} . Corn-steep liquor was found to contain phenylethylamine which was preferentially incorporated into the penicillin molecule to yield benzyl penicillin (Penicillin G). Having established that the activity of penicillin lay in the side chain, and that the limiting factor was the synthesis of the side chain, it became standard practice to add side-chain precursors to the medium, in particular phenylacetic acid. Smith and Bide (1948) showed that addition of phenylacetic acid and its derivatives to the medium were capable of both increasing penicillin production threefold and to directing biosynthesis towards increasing the proportion of benzyl penicillin from 0 to 93% at the expense of other penicillins. Phenylacetic acid is still the most widely used precursor in penicillin production. Some important examples of precursors are given in Table 4.12.

Inhibitors

When certain inhibitors are added to fermentations, more of a specific product may be produced, or a metabolic intermediate which is normally metabolized is accumulated. One of the earliest examples is the microbial production of glycerol (Eoff *et al.*, 1919). Glycerol production depends on modifying the ethanol fermentation by removing acetaldehyde. The addition of sodium bisulphite to the broth leads to the formation of the acetaldehyde bisulphite addition compound (sodium hydroxy ethyl sulphite). Since acetaldehyde is

TABLE 4.12. Precursors used in fermentation processes

| Precursor | Product | Micro-organism | Reference |
|---|---------------------|---|---------------------------------|
| Phenylacetic-acid related compounds | Penicillin G | <i>Penicillium chrysogenum</i> | Moyer and Coghill and (1947) |
| Phenoxy acetic acid Chloride | Penicillin V | <i>Penicillium chrysogenum</i> | Soper <i>et al.</i> (1948) |
| | Chlortetracycline | <i>Streptomyces aureofaciens</i> | Van Dyck and de Somer (1952) |
| Chloride | Griseofulvin | <i>Penicillium griseofulvin</i> | Rhodes <i>et al.</i> (1955) |
| * Propionate | Riboflavin | <i>Lactobacillus bulgaricus</i> | Smiley and Stone (1955) |
| Cyanides | Vitamin B12 | <i>Propriobacterium</i> , <i>Streptomyces</i> spp. | Mervyn and Smith (1964) |
| β -Iononones | Carotenoids | <i>Phycomyces blakesleeanus</i> | Reyes <i>et al.</i> (1964) |
| α -Amino butyric acid | L-Isoleucine | <i>Bacillus subtilis</i> | Nakayama (1972b) |
| D-Threonine | L-Isoleucine | <i>Serratia marcescens</i> | |
| Anthranoic acid | L-Tryptophan | <i>Hansenula anomala</i> | |
| Nucleosides and bases | Nikkomycins | <i>Streptomyces tendae</i> | |
| Dihydronovobiocin acid | Dihydronovobiocin | <i>Streptomyces</i> sp. | Vecht-Lifshitz and Braun (1989) |
| <i>p</i> -Hydroxycinnamate | Organomycin A and B | <i>Streptomyces organonensis</i> | Walton <i>et al.</i> (1962) |
| DL- α -Amino butyric acid | Cyclosporin A | <i>Tolypocladium inflatum</i> | Eiki <i>et al.</i> (1992) |
| L-Threonine | Cyclosporin C | | Kobel and Traber (1982) |
| Tyrosine or <i>p</i> -hydroxy-phenylglycine | Dimethylvancamycin | <i>Nocardia orientalis</i> | Boeck <i>et al.</i> (1984) |

* Yields are not so high as by other techniques

no longer available for re-oxidation of NADH_2 , its place as hydrogen acceptor is taken by dihydroacetone phosphate, produced during glycolysis. The product of this reaction is glycerol-3-phosphate, which is converted to glycerol.

The application of general and specific inhibitors are illustrated in Table 4.13. In most cases the inhibitor is effective in increasing the yield of the desired product and reducing the yield of undesirable related products. A number of studies have been made with potential chlorination inhibitors, e.g. bromide, to minimize chlortetracycline production during a tetracycline fermentation (Gourevitch *et al.*, 1956; Lepetit S.p.A., 1957; Goodman *et al.*, 1959; Lein *et al.*, 1959; Szumski, 1959).

Inhibitors have also been used to affect cell-wall structure and increase the permeability for release of metabolites. The best example is the use of penicillin and surfactants in glutamic acid production (Phillips and Somerson, 1960).

Inducers

The majority of enzymes which are of industrial interest are inducible. Induced enzymes are synthesized only in response to the presence in the environment of an inducer. Inducers are often substrates such as starch

or dextrins for amylases, maltose for pullulanase and pectin for pectinases. Some inducers are very potent, such as isovaleronitrile inducing nitrilase (Kobayashi *et al.*, 1992). Substrate analogues that are not attacked by the enzyme may also serve as enzyme inducers. Most inducers which are included in microbial enzyme media (Table 4.14) are substrates or substrate analogues, but intermediates and products may sometimes be used as inducers. For example, maltodextrins will induce amylase and fatty acids induce lipase. However, the cost may prohibit their use as inducers in a commercial process. Reviews have been published by Aunstrup *et al.* (1979) and Demain (1990).

One unusual application of an inducer is the use of yeast mannan in streptomycin production (Inamine *et al.*, 1969). During the fermentation varying amounts of streptomycin and mannosidostreptomycin are produced. Since mannosidostreptomycin has only 20% of the biological activity of streptomycin, the former is an undesirable product. The production organism *Streptomyces griseus* can be induced by yeast mannan to produce β -mannosidase which will convert mannosidostreptomycin to streptomycin.

It is now possible to produce a number of heterologous proteins in yeasts, fungi and bacteria. These include proteins of viral, human, animal, plant and mi-

TABLE 4.13. Specific and general inhibitors used in fermentations

| Product | Inhibitor | Main effect | Micro-organism | Reference |
|----------------------------------|--------------------------------------|---|----------------------------------|--------------------------------|
| Glycerol | Sodium bisulphite | Acetaldehyde production repressed | <i>Saccharomyces cerevisiae</i> | Eoff <i>et al.</i> (1919) |
| Tetracycline | Bromide | Chlortetracycline formation repressed | <i>Streptomyces aureofaciens</i> | Lepetit (1957) |
| Glutamic acid | Penicillin | Cell wall permeability | <i>Micrococcus glutamicus</i> | Phillips <i>et al.</i> (1960) |
| Citric acid | Alkali metal/phosphate, pH below 2.0 | Oxalic acid repressed | <i>Aspergillus niger</i> | Batti (1967) |
| Valine | Various inhibitors | Various effects with different inhibitors | <i>Brevibacterium roseum</i> | Uemura <i>et al.</i> (1972) |
| Rifamycin B | Di-ethyl barbiturate | Other rifamycins inhibited | <i>Nocardia mediterranei</i> | Lancini and White (1973) |
| 7-Chloro-6 de-methyltetracycline | Ethionine | Affects one-carbon transfer reactions | <i>Streptomyces aureofaciens</i> | Neidleman <i>et al.</i> (1963) |

icrobial origin (Peberdy, 1988; Wayne Davies, 1991). However, heterologous proteins may show some degree of toxicity to the host and have a major influence on the stability of heterologous protein expression. As well as restricting cell growth as biomass the toxicity will provide selective conditions for segregant cells which no longer synthesize the protein at such a high level (Goodey *et al.*, 1987). Therefore, optimum growth conditions may be achieved by not synthesizing a heterologous protein continuously and only inducing it after the host culture has grown up in a vessel to produce sufficient biomass (Piper and Kirk, 1991). In cells of *S. cerevisiae* where the *Gal1* promoter is part of the gene expression system, product formation may be induced

by galactose addition to the growth medium which contains glycerol or low non-repressing levels of glucose as a carbon source.

One commercial system that has been developed is based on the *alcA* promoter in *Aspergillus nidulans* to express human interferon $\alpha 2$ (Wayne Davies, 1991). This can be induced by volatile chemicals, such as ethylmethyl ketone, which are added when biomass has increased to an adequate level and the growth medium contains a non-repressing carbon source or low non-repressing levels of glucose.

Methyotrophic yeasts such as *Hansenula polymorpha* and *Pichia pastoris* may be used as alternative systems because of the presence of an alcohol oxidase

TABLE 4.14. Some examples of industrially important enzyme inducers

| Enzyme | Inducer | Micro-organism | Reference |
|-----------------------|--|---|---------------------------------|
| α -Amylase | Starch | <i>Aspergillus</i> spp. | Windish and Mhatre (1965) |
| | Maltose | <i>Bacillus subtilis</i> | |
| Pullulanase | Maltose | <i>Aerobacter aerogenes</i> | Wallenfels <i>et al.</i> (1966) |
| α -Mannosidase | Yeast mannos | <i>Streptomyces griseus</i> | Inamine <i>et al.</i> (1969) |
| Penicillin acylase | Phenylacetic acid | <i>Escherichia coli</i> | Carrington (1971) |
| Proteases | Various proteins | <i>Bacillus</i> spp. <i>Streptococcus</i> spp. <i>Streptomyces</i> spp. <i>Aspergillus</i> spp. <i>Mucor</i> spp. | Keay (1971) Aunstrup (1974) |
| Cellulase | Cellulose | <i>Trichoderma viride</i> | Reese (1972) |
| Pectinases | Pectin (beet pulp, apple pomace, citrus peel) | <i>Aspergillus</i> spp. | Fogarty and Ward (1974) |
| Nitrilase | Isovaleronitrile | <i>Rhodococcus rhodochrous</i> | Kobayashi <i>et al.</i> (1992) |

promoter (Veale and Sudbery, 1991). During growth on methanol, which also acts as an inducer, the promoter is induced to produce about 30% of the cell protein. In the presence of glucose or ethanol, it is undetectable. Expression systems have been developed with *P. pastoris* for tumour necrosis factor, hepatitis B surface antigen and α -galactosidase. Hepatitis B surface antigen and other heterologous proteins can also be expressed by *H. polymorpha*.

OXYGEN REQUIREMENTS

It is sometimes forgotten that oxygen, although not added to an initial medium as such, is nevertheless a very important component of the medium in many processes, and its availability can be extremely important in controlling growth rate and metabolite production. This will be discussed in detail in Chapter 9.

The medium may influence the oxygen availability in a number of ways including the following:

1. **Fast metabolism.** The culture may become oxygen limited because sufficient oxygen cannot be made available in the fermenter if certain substrates, such as rapidly metabolized sugars which lead to a high oxygen demand, are available in high concentrations.
2. **Rheology.** The individual components of the medium can influence the viscosity of the final medium and its subsequent behaviour with respect to aeration and agitation.

3. **Antifoams.** Many of the antifoams in use will act as surface active agents and reduce the oxygen transfer rate. This topic will be considered in a later section of this chapter.

Fast metabolism

Nutritional factors can alter the oxygen demand of the culture. *Penicillium chrysogenum* will utilize glucose more rapidly than lactose or sucrose, and it therefore has a higher specific oxygen uptake rate when glucose is the main carbon source (Johnson, 1946). Therefore, when there is the possibility of oxygen limitation due to fast metabolism, it may be overcome by reducing the initial concentration of key substrates in the medium and adding additional quantities of these substrates as a continuous or semi-continuous feed during the fermentation (see Tables 4.1. and 4.15; Chapters 2 and 9). It can also be overcome by changing the composition of the medium, incorporating higher carbohydrates (lactose, starch, etc.) and proteins which are not very rapidly metabolized and do not support such a large specific oxygen uptake rate.

Rheology

Deindoerfer and West (1960) reported that there can be considerable variation in the viscosity of compounds that may be included in fermentation media.

TABLE 4.15. Some processes using batch feed or continuous feed or in which they have been tried

| Product | Additions | Reference |
|-------------------------|--------------------------------------|-----------------------------------|
| Yeast | Molasses, nitrogen sources, P and Mg | Harrison (1971) |
| Glycerol | Sugar, Na_2CO_3 | Reed and Peppler (1973) |
| Acetone-butyl alcohol | Additions and withdrawals of wort | Eoff <i>et al.</i> (1919) |
| Riboflavin | Carbohydrate | Soc. Richard <i>et al.</i> (1921) |
| Penicillin | Glucose and NH_3 | Moss and Klein (1946) |
| Novobiocin | Various carbon and nitrogen sources | Hosler and Johnson (1953) |
| Griseofulvin | Carbohydrate | Smith (1956) |
| Rifamycin | Glucose, fatty acids | Hockenhull (1956) |
| Gibberellins | Glucose | Pan <i>et al.</i> (1959) |
| Vitamin B_{12} | Glucose | Borrow <i>et al.</i> (1960) |
| Tetracyclines | Glucose | Becher <i>et al.</i> (1961) |
| Citric acid | Carbohydrates, NH_3 | Avanzini (1963) |
| Single-cell protein | Methanol | Shepherd (1963) |
| Candidin | Glucose | Harrison <i>et al.</i> (1972) |
| Streptomycin | Glucose, ammonium sulphate | Martin and McDaniel (1975) |
| Cephalosporin | Fresh medium addition | Singh <i>et al.</i> (1976) |
| | | Trilli <i>et al.</i> (1977) |

Polymers in solution, particularly starch and other polysaccharides, may contribute to the rheological behaviour of the fermentation broth (Tuffile and Pinho, 1970). As the polysaccharide is degraded, the effects on rheological properties will change. Allowances may also have to be made for polysaccharides being produced by the micro-organism (Banks *et al.*, 1974; Leduy *et al.*, 1974). This aspect is considered in more detail in Chapter 9.

ANTIFOAMS

In most microbiological processes, foaming is a problem. It may be due to a component in the medium or some factor produced by the micro-organism. The most common cause of foaming is due to proteins in the medium, such as corn-steep liquor, Pharmamedia, peanut meal, soybean meal, yeast extract or meat extract (Schugerl, 1985). These proteins may denature at the air–broth interface and form a skin which does not rupture readily. The foaming can cause removal of cells from the medium which will lead to autolysis and the further release of microbial cell proteins will probably increase the stability of the foam. If uncontrolled, then numerous changes may occur and physical and biological problems may be created. These include reduction in the working volume of the fermenter due to oxygen-exhausted gas bubbles circulating in the system (Lee and Tyman, 1988), changes in bubble size, lower mass and heat transfer rates, invalid process data due to interference at sensing electrodes and incorrect monitoring and control (Vardar-Sukan, 1992). The biological problems include deposition of cells in upper parts of the fermenter, problems of sterile operation with the air filter exits of the fermenter becoming wet, and there is danger of microbial infection and the possibility of siphoning leading to loss of product.

Hall *et al.* (1973) have recognized five patterns of foaming in fermentations:

1. Foaming remains at a constant level through-out the fermentation. Initially it is due to the medium and later due to microbial activity.
2. A steady fall in foaming during the early part of the fermentation, after which it remains constant. Initially it is due to the medium but there are no later effects caused by the micro-organism.
3. The foaming falls slightly in the early stages of the fermentation then rises. There are very slight effects caused by the medium but the major effects are due to microbial activity.

4. The fermentation has a low initial foaming capacity which rises. These effects are due solely to microbial activity.
5. A more complex foaming pattern during the fermentation which may be a combination of two or more of the previously described patterns.

If excessive foaming is encountered there are three ways of approaching the problem:

1. To try and avoid foam formation by using a defined medium and a modification of some of the physical parameters (pH, temperature, aeration and agitation). This assumes that the foam is due to a component in the medium and not a metabolite.
2. The foam is unavoidable and antifoam should be used. This is the more standard approach.
3. To use a mechanical foam breaker. (See Chapter 7.)

Antifoams are surface active agents, reducing the surface tension in the foams and destabilizing protein films by (a) hydrophobic bridges between two surfaces, (b) displacement of the absorbed protein, and (c) rapid spreading on the surface of the film (Van't Riet and Van Sonsbeck, 1992). Other possible mechanisms have been discussed by Ghildyal *et al.* (1988), Lee and Tyman (1988) and Vardar-Sukan (1992).

An ideal antifoam should have the following properties:

1. Should disperse readily and have fast action on an existing foam.
2. Should be active at low concentrations.
3. Should be long acting in preventing new foam formation.
4. Should not be metabolized by the micro-organism.
5. Should be non-toxic to the micro-organism.
6. Should be non-toxic to humans and animals.
7. Should not cause any problems in the extraction and purification of the product.
8. Should not cause any handling hazards.
9. Should be cheap.
10. Should have no effect on oxygen transfer.
11. Should be heat sterilizable.

The following compounds which meet most of these requirements have been found to be most suitable in different fermentation processes (Solomons, 1969; Ghildyal *et al.*, 1988):

1. Alcohols; stearyl and octyl decanol.
2. Esters.
3. Fatty acids and derivatives, particularly glycerides, which include cottonseed oil, linseed oil, soy-bean oil, olive oil, castor oil, sunflower oil, rapeseed oil and cod liver oil.
4. Silicones.
5. Sulphonates.
6. Miscellaneous; Alkaterge C, oxazaline, poly-propylene glycol.

These antifoams are generally added when foaming occurs during the fermentation. Because many antifoams are of low solubility they need a carrier such as lard oil, liquid paraffin or castor oil, which may be metabolized and affect the fermentation process (Solomons, 1967).

Unfortunately, the concentrations of many antifoams which are necessary to control fermentations will reduce the oxygen-transfer rate by as much as 50%; therefore antifoam additions must be kept to an absolute minimum. There are also other antifoams which will increase the oxygen-transfer rate (Ghildyal *et al.*, 1988). If the oxygen-transfer rate is severely affected by antifoam addition then mechanical foam breakers may have to be considered as a possible alternative. Vardar-Sukan (1992) concluded that foam control in industry is still an empirical art. The best method for a particular process in one factory is not necessarily the best for the same process on another site. The design and operating parameters of a fermenter may affect the properties and quantity of foam formed.

MEDIUM OPTIMIZATION

At this stage it is important to consider the optimization of a medium such that it meets as many as possible of the seven criteria given in the introduction to this chapter. The meaning of optimization in this context does need careful consideration (Winkler, 1991). When considering the biomass growth phase in isolation it must be recognized that efficiently grown biomass produced by an 'optimized' high productivity growth phase is not necessarily best suited for its ultimate purpose, such as synthesizing the desired product. Different combinations and sequences of process conditions need to be investigated to determine the growth conditions which produce the biomass with the physiological state best constituted for product formation. There may be a sequence of phases each with a specific set of optimal conditions.

Medium optimization by the classical method of changing one independent variable (nutrient, antifoam, pH, temperature, etc.) while fixing all the others at a certain level can be extremely time consuming and expensive for a large number of variables. To make a full factorial search which would examine each possible combination of independent variable at appropriate levels could require a large number of experiments, x^n , where x is the number of levels and n is the number of variables. This may be quite appropriate for three nutrients at two concentrations (2^3 trials) but not for six nutrients at three concentrations. In this instance 3^6 (729) trials would be needed. Industrially the aim is to perform the minimum number of experiments to determine optimal conditions. Other alternative strategies must therefore be considered which allow more than one variable to be changed at a time. These methods have been discussed by Stowe and Mayer (1966), McDaniel *et al.* (1976), Hendrix (1980), Nelson (1982), Greasham and Inamine (1986), Bull *et al.* (1990) and Hicks (1993).

When more than five independent variables are to be investigated, the Plackett-Burman design may be used to find the most important variables in a system, which are then optimized in further studies (Plackett and Burman, 1946). These authors give a series of designs for up to one hundred experiments using an experimental rationale known as balanced incomplete blocks. This technique allows for the evaluation of $X - 1$ variables by X experiments. X must be a multiple of 4, e.g. 8, 12, 16, 20, 24, etc. Normally one determines how many experimental variables need to be included in an investigation and then selects the Plackett-Burman design which meets that requirement most closely in multiples of 4. Any factors not assigned to a variable can be designated as a dummy variable. Alternatively, factors known to not have any effect may be included and designated as dummy variables. As will be shown shortly in a worked example (Table 4.16), the incorporation of dummy variables into an experiment makes it possible to estimate the variance of an effect (experimental error).

Table 4.16 shows a Plackett-Burman design for seven variables (*A-G*) at high and low levels in which two factors, *E* and *G*, are designated as 'dummy' variables. These can then be used in the design to obtain an estimate of error. Normally three dummy variables will provide an adequate estimate of the error. However, more can be used if fewer real variables need to be studied in an investigation (Stowe and Mayer, 1966). Each horizontal row represents a trial and each vertical

TABLE 4.16. Plackett-Burman design for seven variables (Nelson, 1982)

| Trial | Variables | | | | | | Yield | |
|-------|-----------|---|---|---|---|---|-------|-----|
| | A | B | C | D | E | F | | |
| 1 | H | H | H | L | H | L | H | 1.1 |
| 2 | L | H | H | H | L | H | L | 6.3 |
| 3 | L | L | H | H | H | L | H | 1.2 |
| 4 | H | L | L | H | H | H | L | 0.8 |
| 5 | L | H | L | L | H | H | H | 6.0 |
| 6 | H | L | H | L | L | H | H | 0.9 |
| 7 | H | H | L | H | L | L | H | 1.1 |
| 8 | L | L | L | L | L | L | L | 1.4 |

H denotes a high level value; L denotes a low level value.

column represents the H (high) and L (low) values of one variable in all the trials. This design (Table 4.16) requires that the frequency of each level of a variable in a given column should be equal and that in each test (horizontal row) the number of high and low variables should be equal. Consider the variable *A*; for the trials in which *A* is high, *B* is high in two of the trials and low in the other two. Similarly, *C* will be high in two trials and low in two, as will all the remaining variables. For those trials in which *A* is low, *B* will be high two times and low two times. This will also apply to all the other variables. Thus, the effects of changing the other variables cancel out when determining the effect of *A*. The same logic then applies to each variable. However, no changes are made to the high and low values for the *E* and *G* columns. Greasham and Inamine (1986) state that although the difference between the levels of each variable must be large enough to ensure that the optimum response will be included, caution must be taken when setting the level differential for sensitive variables, since a differential that is too large could mask the other variables. The trials are carried out in a randomized sequence.

The effects of the dummy variables are calculated in the same way as the effects of the experimental variables. If there are no interactions and no errors in measuring the response, the effect shown by a dummy variable should be 0. If the effect is not equal to 0, it is assumed to be a measure of the lack of experimental precision plus any analytical error in measuring the response (Stowe and Mayer, 1966).

This procedure will identify the important variables and allow them to be ranked in order of importance to decide which to investigate in a more detailed study to determine the optimum values to use.

The stages in analysing the data (Tables 4.16 and 4.17) using Nelson's (1982) example are as follows:

1. Determine the difference between the average of the H (high) and L (low) responses for each independent and dummy variable.

Therefore the difference =

$$\Sigma A(H) - \Sigma A(L).$$

The effect of an independent variable on the response is the difference between the average

TABLE 4.17. Analysis of the yields shown in Table 4.16 (Nelson, 1982)

| | Factor | | | | | | |
|---------------------------|------------------------------------|--------|-------|-------|-------|--------|-------|
| | A | B | C | D | E | F | G |
| $\Sigma(H)$ | 3.9 | 14.5 | 9.5 | 9.4 | 9.1 | 14.0 | 9.2 |
| $\Sigma(L)$ | 14.9 | 4.3 | 9.3 | 9.4 | 9.7 | 4.8 | 9.6 |
| Difference | -11.0 | 10.2 | 0.2 | 0.0 | -0.6 | 9.2 | -0.4 |
| Effect | -2.75 | 2.55 | 0.05 | 0.00 | -0.15 | 2.30 | -0.10 |
| Mean square | 15.125 | 13.005 | 0.005 | 0.000 | 0.045 | 10.580 | 0.020 |
| Mean square for 'error' = | $\frac{0.045 + 0.020}{2} = 0.0325$ | | | | | | |

response for the four experiments at the high level and the average value for four experiments at the low level.

Thus the effect of

$$A = \frac{\sum A(H)}{4} - \frac{\sum A(L)}{4}$$

$$= \frac{2(\sum A(H) - \sum A(L))}{8}$$

This value should be near zero for the dummy variables.

2. Estimate the mean square of each variable (the variance of effect).

For A the mean square will be =

$$\frac{(\sum A(H) - \sum A(L))^2}{8}$$

3. The experimental error can be calculated by averaging the mean squares of the dummy effects of E and G .

Thus, the mean square for error =

$$\frac{0.045 + 0.020}{2} = 0.0325$$

This experimental error is not significant.

4. The final stage is to identify the factors which are showing large effects. In the example this was done using an F -test for

$$\frac{\text{Factor mean square.}}{\text{error mean square.}}$$

This gives the following values:

$$A = \frac{15.125}{0.0325} = 465.4,$$

$$B = \frac{13.005}{0.0325} = 400.2,$$

$$C = \frac{0.0500}{0.0325} = 3.255$$

$$D = \frac{0.0000}{0.0325} = 0.00$$

$$F = \frac{10.580}{0.0325} = 325.6.$$

When Probability Tables are examined it is found that Factors A , B and F show large effects which are very significant, whereas C shows a very low effect which is not significant and D shows no effect. A , B and F have been identified as the most important

factors. The next stage would then be the optimization of the concentration of each factor, which will be discussed later.

Nelson (1982) has also referred to the possibility of two factor interactions which might occur when designing Table 4.16. This technique has also been discussed by McDaniel *et al.* (1976), Greasham and Inamine (1986), Bull *et al.* (1990) and Hicks (1993).

The next stage in medium optimization would be to determine the optimum level of each key independent variable which has been identified by the Plackett-Burman design. This may be done using response surface optimization techniques which were introduced by Box and Wilson (1951). Hendrix (1980) has given a very readable account of this technique and the way in which it may be applied. Response surfaces are similar to contour plots or topographical maps. Whilst topographical maps show lines of constant elevation, contour plots show lines of constant value. Thus, the contours of a response surface optimization plot show lines of identical response. In this context, response means the result of an experiment carried out at particular values of the variables being investigated.

The axes of the contour plot are the experimental variables and the area within the axes is termed the response surface. To construct a contour plot, the results (responses) of a series of experiments employing different combinations of the variables are inserted on the surface of the plot at the points delineated by the experimental conditions. Points giving the same results (equal responses) are then joined together to make a contour line. In its simplest form two variables are examined and the plot is two dimensional. It is important to appreciate that both variables are changed in the experimental series, rather than one being maintained constant, to ensure that the data are distributed

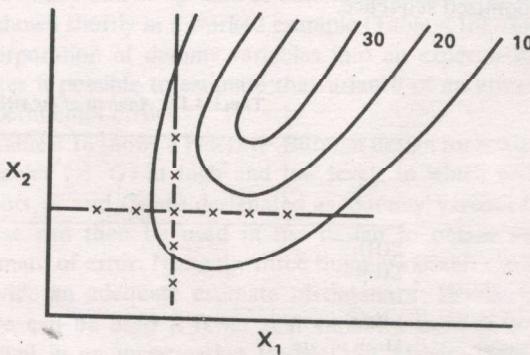


FIG. 4.1. Optimal point of a response surface by one factor at a time.

over the response surface. In Fig. 4.1, the profile generated by fixing X_1 and changing X_2 and then using the best X_2 value and changing X_1 constitutes a cross which may not encroach upon the area in which the optimum resides.

The technique may be applied at different levels of sophistication. Hendrix applied the technique at its simplest level to predict the optimum combination of two variables. The values of the variables for the initial experiments are chosen randomly or with the guidance of previous experience of the process. There is little to be gained from using more than 15–20 experiments. The resulting contour map gives an indication of the area in which the optimum combination of variables resides. A new set of experiments may then be designed within the indicated zone. Hendrix proposed the following strategy to arrive at the optimum in an incremental fashion:

1. Define the space on the plot to be explored.
2. Run five random experiments in this space.
3. Define a new space centred upon the best of the five experiments and make the new space smaller than the previous one, perhaps by cutting each dimension by one half.
4. Run five more random experiments in this new space.
5. Continue doing this until no further improvement is observed, or until you cannot afford any more experiments!

The more sophisticated applications of the response surface technique use mathematical models to analyse the first round of experimental data and to predict the relationship between the response and the variables. These calculations then allow predictive contours to be drawn and facilitate a more rapid optimization with fewer experiments. If three or more variables are to be examined then several contour maps will have to be constructed. Hicks (1993) gives an excellent account of the development of equations to model the different interactions which may take place between the variables. Several computer software packages are now available which allow the operator to determine the equations underlying the responses and, thus, to determine the likely area on the surface in which the optimum resides. Some examples of the types of response surface profiles that may be generated are illustrated in Fig. 4.2.

The following examples illustrate the application of the technique:

- (i) McDaniel *et al.* (1976), Fig. 4.3. The variables under investigation were cereose and soybean level, with the analysis indicating the optimum to be 6.2% cereose and 3.2% soybean.
- (ii) Saval *et al.* (1993). The medium for streptomycin production was optimized for four components resulting in a 52% increase in streptomycin yield, a 10% increase in mycelial dry weight and a 48% increase in specific growth rate (Table 4.18).

When further optimization experiments are necessary for medium development in large vessels, the number of experiments will normally be restricted because of the cost and the lack of spare large vessels (Spendley *et al.*, 1962). The simplex search method attempts to optimize n variables by initially performing

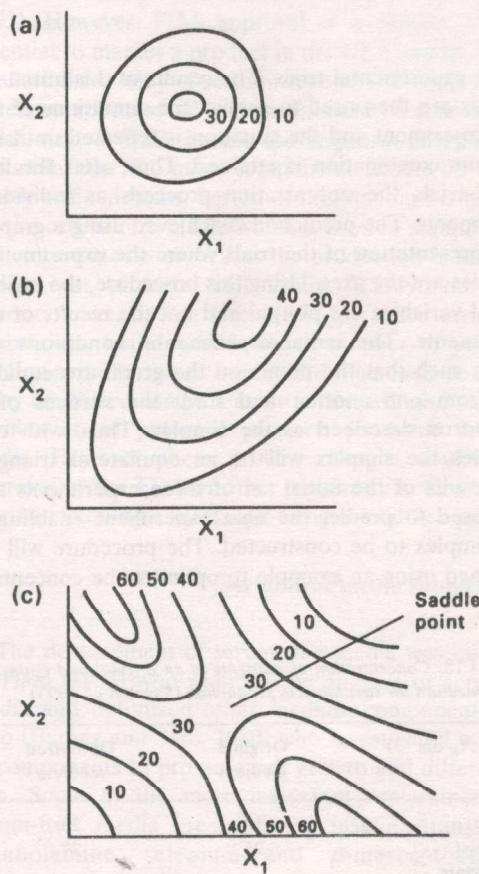


FIG. 4.2. Typical response surfaces in two dimensions; (a) mound, (b) rising ridge, (c) saddle.

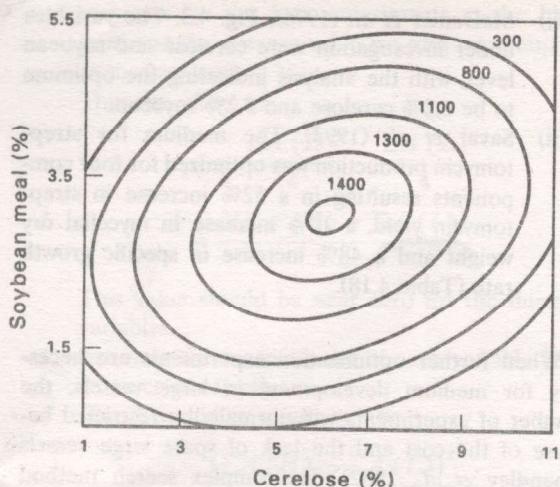


FIG. 4.3. Contour plot of two independent variables, cerelose and soybean meal, for optimization of the candidin fermentation (Redrawn from McDaniel *et al.*, 1976; Bull *et al.*, 1990).

$n + 1$ experimental trials. The results of this initial set of trials are then used to predict the conditions of the next experiment and the situation is repeated until the optimum combination is attained. Thus, after the first set of trials the optimization proceeds as individual experiments. The prediction is achieved using a graphical representation of the trials where the experimental variables are the axes. Using this procedure, the experimental variables are plotted and not the results of the experiments. The initial experimental conditions are chosen such that the points on the graph are equidistant from one another and form the vertices of a polyhedron described as the simplex. Thus, with two variables the simplex will be an equilateral triangle. The results of the initial set of three experiments are then used to predict the next experiment enabling a new simplex to be constructed. The procedure will be explained using an example to optimize the concentra-

TABLE 4.18. Concentration of nutrients in an original and optimized medium for streptomycin production (Saval *et al.* 1993)

| Nutrient (g dm ⁻³) | Original medium | Optimized medium |
|---------------------------------|-----------------|------------------|
| Glucose | 10 | 23 |
| Beer-yeast autolysate | 25 | 27 |
| NaCl | 10 | 8 |
| K ₂ HPO ₄ | 1 | 1 |

tions of carbon and nitrogen sources in a medium for antibiotic production.

In our example a graph is constructed in which the x axis represents the concentration range of the carbon source (the first variable) and the y axis represents the concentration range of the nitrogen source (the second variable). The first vertex A (experimental point) of the simplex represents the current concentrations of the two variables which are producing the best yield of the antibiotic. The experiment for the second vertex B is planned using a new carbon-nitrogen mixture and the position of the third vertex C can now be plotted on the graph using lengths AC and BC equal to AB (the simplex equilateral triangle, Fig. 4.4a). The concentrations of the carbon and nitrogen sources to use in the third experiment can now be determined graphically and the experiment can be undertaken to determine the yield of antibiotic. The results of the three experiments are assessed and the worst response to antibiotic production identified. In our example, experiment A was the worst and B the best. The simplex design is

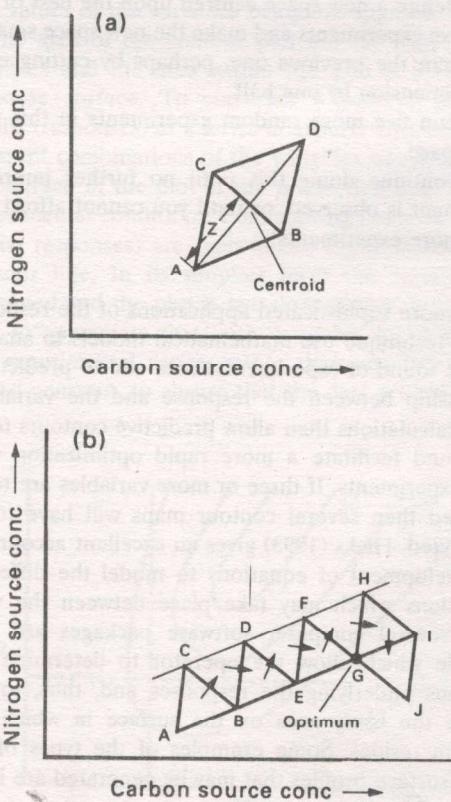


FIG. 4.4. (a) Simplex optimization for a pair of independent variables (with reflection). (b) Simplex optimization of pair of independent variables which has reached the optimum.

now used to design the next experiment. A new simplex (equilateral triangle) BCD is constructed opposite the worst response (i.e. A) using the existing vertices B and C. A line is drawn from A through the centroid (mid point) of BC. D (the next experiment) will be on this line and the sides BD and CD will be the same length as BC. This process of constructing the new simplex is described as reflection. Once the position of D is known, the concentrations of the carbon and nitrogen sources can be determined graphically, the experiment performed and the production of antibiotic assayed. Thus, a series of simplexes can be constructed moving in a crabwise way. The procedure is continued until the optimum is located. At this point the simplex begins to circle on its self, indicating the optimum concentration (Fig 4.4b; Greasham and Inamine, 1986). However, if a new vertex exhibits the lowest response, the simplex would reflect back on to the previous one, halting movement towards the optimum. In this case the new simplex is constructed opposite the second least desirable response using the method previously described.

If it is decided that the supposed optimum should be reached more rapidly then the distance z between the centroid and D may be increased (expanded) by a factor which is often 2. If the optimum is thought to have nearly been reached then the distance z may be decreased by a factor of 0.5 (contraction). This modified simplex optimization was first proposed by Nelder and Mead (1965) and has been discussed by Greasham and Inamine (1986).

The simplex method may also be used in small scale media development experiments to help identify the possible optimum concentration ranges to test in more extensive multifactorial experiments.

Animal cell media

Mammalian cell lines have been cultured *in vitro* for 40 years. Initially, animal cells were required for vaccine manufacture but they are now also used in the production of monoclonal antibodies, interferon, etc. The media initially used for this purpose contained about 10% serum (foetal calf or calf) plus other organic and inorganic components. Since this pioneering work it has been possible to develop a range of serum-free media (Ham, 1965; Barnes and Sato, 1980). These media contain carbohydrates, amino acids, vitamins, nucleic acids, etc, dissolved in high purity water. Media costs are therefore considerably higher than those for microbial cells. At a 1000 dm³ scale the medium costs

may account for 40% of the unit costs, and serum may be 80% of the medium cost (Wilkinson, 1987).

Serum

The serum is a very complex mixture containing approximately 1000 components including inorganic salts, amino acids, vitamins, carbon sources, hormones, growth factors, haemoglobin, albumin and other compounds (Brooks, 1975; Glassy *et al.*, 1988). However, most of them do not appear to be needed for growth and differentiation of cell lines which have been tested (Barnes and Sato, 1980; Darfler and Insel, 1982).

Serum is used extensively in production media for animal cell culture to produce recombinant proteins and antibody based products for *in vivo* use in humans. At present the regulations governing the quality of serum which can be used for manufacturing processes vary considerably from country to country (Hodgson, 1993). However, FDA approval of a process will be essential to market a product in the USA and therefore regulate the quality of serum which can be used. Serum tested by approved laboratories should be free of bacterial, viral or BSE (bovine sporangiform encephalitis) contamination and other components should be within strictly defined limits. Serum of this standard is needed for the cell culture media which is used to maintain the cell culture stocks as well as the production media.

The cost of foetal calf serum, US\$190 dm⁻³ in Europe, makes serum free media attractive economic alternatives, but it would take a number of years to develop suitable serum free media. The absence of the many unutilized components in serum will also simplify purification of potential products produced in such media. However, these process changes would need approval by the FDA or other regulatory bodies before a product could be marketed using a modified process.

Serum-free media supplements

The development of serum-free media was initiated by Ham (1965) who reduced the amount of serum in media and optimized other medium components and Sato (Barnes and Sato, 1980) who investigated a range of components to promote cell growth and differentiation. Some of the more important replacements in serum-free media are albumin, insulin, transferrin, ethanolamine, selenium and β -mercaptoethanol (Glassy *et al.*, 1988).

The advantages of removing serum from media include:

1. More consistent and definable medium composition to reduce batch variation.
2. Reduction in potential contamination to make sterility easier to achieve.
3. Potential cost savings because of cheaper replacement components.
4. Simplifying downstream processing because the total protein content of the medium has been reduced.

Protein-free media

The elimination of proteins seems an attractive objective. However, the design of such media is difficult and their use may be very limited and not very cost effective. Hamilton and Ham (1977) demonstrated the growth of Chinese hamster cell lines in a protein-free medium formulated from amino acids, vitamins, organic compounds and inorganic salts. Other media have been developed by Cleveland *et al.* (1983) and Shive *et al.* (1986).

Trace elements

The role of trace elements in medium formulation can be significant. Cultured cells normally require Fe, Zn, Cu, Se, Mn, Mo and V (Ham and McKeehan, 1975). These are often present as impurities in other media components. Cleveland *et al.* (1983) found that if the number of trace elements were increased, insulin, transferrin, albumin and liposomes were not needed in a serum-free hybridoma medium. They included Al, Ag, Ba, Br, Cd, Co, Cr, F, Ge, J, Rb, Zr, Si, Ni and Sn as well as those previously mentioned.

Osmolality

The optimum range of osmotic pressure for growth is often quite narrow and varies with the type of cell and the species from which it was isolated. It may be necessary to adjust the concentration of NaCl when major additions are made to a medium.

pH

The normal buffer system in tissue culture media is the CO₂-bicarbonate system. This is a weak buffering system and can be improved by the use of a zwitterionic buffer such as Hepes, either in addition to or instead of the CO₂-bicarbonate buffer. Continuous pH control is achieved by the addition of sodium bicarbonate or sodium hydroxide (with fast mixing) when too acid. The pH does not normally become too alkaline so acid additions are not required but provision may be made for CO₂ additions (Fleischaker, 1987).

Non-nutritional media supplements

Sodium carboxy methyl cellulose may be added to media at 0.1% to help to minimize mechanical damage caused by the shear force generated by the stirrer impeller. The problems of foam formation and subsequent cell damage and losses can affect animal cell growth. Pluronic F-68 (polyglycol) can provide a protective effect to animal cells in stirred and sparged vessels. In media which are devoid of Pluronic F-68, cells may become more sensitive to direct bubble formation in the presence of an antifoam agent being used to suppress foam formation (Zhang *et al.*, 1992).

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