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### Media formulation

Most fermentations require liquid media, often referred to as broth, although some solid-substrate fermentations are operated. Fermentation media must satisfy all the nutritional requirements of the microorganism and fulfil the technical objectives of the process. The nutrients should be formulated to promote the synthesis of the target product, either cell biomass or a specific metabolite. In most industrial fermentation processes there are several stages where media are required. They may include several inoculum (starter culture) propagation steps, pilot-scale fermentations and the main production fermentation. The technical objectives of inoculum propagation and the main fermentation are often very different, which may be reflected in differences in their media formulations. Where biomass or primary metabolites are the target product, the objective is to provide a production medium that allows optimal growth of the microorganism. For secondary metabolite production, such as antibiotics, their biosynthesis is not growth related. Consequently, for this purpose, media are designed to provide an initial period of cell growth, followed by conditions optimized for secondary metabolite production. At this point the supply of one or more nutrients (carbon, phosphorus or nitrogen source) may be limited and rapid growth ceases.

Most fermentations, except those involving solid substrates, require large quantities of water in which the medium is formulated. General media requirements include a carbon source, which in virtually all industrial fermentations provides both energy and carbon units for biosynthesis, and sources of nitrogen, phosphorus and sulphur. Other minor and trace elements must also be supplied, and some microorganisms require added vitamins, such as biotin and riboflavin. Aerobic fermentations are dependent on a continuous input of molecular oxygen, and even some anaerobic fermentations require initial aeration of media, e.g. beer fermenta-

tions. Usually, media incorporate buffers, or the pH is controlled by acid and alkali additions, and antifoam agents may be required. For some processes, precursor, inducer or inhibitor compounds must be introduced at certain stages of the fermentation.

The initial step in media formulation is the examination of the overall process based on the stoichiometry for growth and product formation. This primarily involves consideration of the input of the carbon and nitrogen sources, minerals and oxygen, and their conversion to cell biomass, metabolic products, carbon dioxide, water and heat. From this information it should be possible to calculate the minimum quantities of each element required to produce a certain quantity of biomass or metabolite. Typically, the main elemental formula of microbial cells is approximately  $C_4H_7O_2N$ , which on a basis of dry weight is 48% C, 7% H, 32% O and 14% N. The elemental composition of baker's yeast, for example, is  $C_{3.72}H_{6.11}O_{1.95}N_{0.61}S_{0.017}P_{0.035}K_{0.056}$ . Elemental composition varies slightly with growth rate, but the range is relatively small compared with interspecies differences, particularly between bacteria and fungi. Ideally, a knowledge of the complete elemental composition of the specific industrial microorganism allows further media refinement. This ensures that no element is limiting, unless this is desired for a specific purpose.

Once the elemental requirements of a microorganism have been established, suitable nutrient sources can be incorporated into the media to fulfil these demands. However, it is important to be aware of potential problems that can arise when using certain compounds. For example, those that are rapidly metabolized may repress product formation. To overcome this, intermittent or continuous addition of fresh medium may be carried out to maintain a relatively low concentration that is not repressive. Certain media nutrients or environmental conditions may affect not only the physiology and biochemistry, but also the morphology of the microorganism. In some yeasts the single cells may develop into

pseudo-mycelium or flocculate, and filamentous fungi may form pellets. This may or may not be desirable, as such morphological changes can influence product yield and other fermentation properties.

The media adopted also depend on the scale of the fermentation. For small-scale laboratory fermentations pure chemicals are often used in well-defined media. However, this is not possible for most industrial-scale fermentation processes, simply due to cost, as media components may account for up to 60-80% of process expenditure. Industrial-scale fermentations primarily use cost-effective complex substrates, where many carbon and nitrogen sources are almost undefinable. Most are derived from natural plant and animal materials, often byproducts of other industries, with varied and variable composition. The effects of such batch-to-batch variations must be determined. Small-scale trials are usually performed with each new batch of substrate, particularly to examine the impact on product yield and product recovery.

The main factors that affect the final choice of individual raw materials are as follows.

- 1 Cost and availability: ideally, materials should be inexpensive, and of consistent quality and year round availability.
- 2 Ease of handling in solid or liquid forms, along with associated transport and storage costs, e.g. requirements for temperature control.
- 3 Sterilization requirements and any potential denaturation problems.
- 4 Formulation, mixing, complexing and viscosity characteristics that may influence agitation, aeration and foaming during fermentation and downstream processing stages.
- 5 The concentration of target product attained, its rate of formation and yield per gram of substrate utilized.
- 6 The levels and range of impurities, and the potential for generating further undesired products during the process.
- 7 Overall health and safety implications.

The final composition of industrial media is not solely the concern of the fermentation stage. Crude substrates provide initial cost savings, but their higher levels of impurities may necessitate more costly and complex recovery and purification steps downstream, and possibly increased waste treatment costs. Also, the physical and chemical properties of the formulated medium can influence the sterilization operations employed. A medium that is easily sterilized with minimum thermal damage is vitally important. Thermal damage not only

reduces the level of specific ingredients, but can also produce potentially inhibitory byproducts that may also interfere with downstream processing. Other media characteristics can affect product recovery and purification, and the ease with which the cells are separated from the spent medium (see Chapter 7).

### Carbon sources

A carbon source is required for all biosynthesis leading to reproduction, product formation and cell maintenance. In most fermentations it also serves as the energy source. Carbon requirements may be determined from the biomass yield coefficient (Y), an index of the efficiency of conversion of a substrate into cellular material.

$$Y_{\text{carbon}} (\text{g/g}) = \frac{\text{biomass produced (g)}}{\text{carbon substrate utilized (g)}}$$

For commercial fermentations the determination of yield coefficients for all other nutrients is usually essential. Each may be determined by conducting a series of batch culture experiments where the specific substrate is the only growth-limiting media component and all other nutrients are in excess (see Chapter 2). By varying the initial concentration of the growth-limiting substrate and then plotting total growth against substrate concentration for each batch, the growth yield (Y) can be estimated. However, the value obtained relates to a specific set of operating conditions; varying pH, temperature, etc., can alter the yield coefficient. Various organisms may exhibit different yield coefficients for the same substrate (Table 5.1), due primarily to the pathway by which the compound is metabolized. Differences can also be seen within an individual. For example, *Saccharomyces cerevisiae* grown on glucose has biomass yield coefficients of 0.56 and 0.12 g/g under aerobic and anaerobic conditions, respectively.

As most carbon substrates also serve as energy sources, the organism's efficiency of both adenosine triphosphate (ATP) generation and its utilization are obviously additional key factors. Often, it is very useful, although rather difficult, to estimate how much ATP is required for growth. However, estimates of  $Y_{\text{ATP}}$  (yield of cells per mole of ATP generated during growth) can be calculated if the metabolism of the organism has been fully elucidated (also see Chapter 14, Single cell protein production).

Carbohydrates are traditional carbon and energy sources for microbial fermentations, although other sources may be used, such as alcohols, alkanes and

ic growth  
 ergillus nidulan  
 ndida utilis  
 cherichia coli  
 chia angusta  
 micillium chr  
 pseudomonas  
 pseudomonas  
 iaccharom  
 aerobic g  
 Moorell  
 Escher  
 Klebs  
 Sacc

	$Y_{\text{glucose}}$	$Y_{\text{ethanol}}$	$Y_{\text{methanol}}$	$Y_{\text{oxalate}}$
<b>Aerobic growth</b>				
<i>Aspergillus nidulans</i>	0.61			
<i>Candida utilis</i>	0.51	0.68		
<i>Escherichia coli</i>	0.52			
<i>Pichia angusta</i>			0.36	
<i>Penicillium chrysogenum</i>	0.43			
<i>Pseudomonas aeruginosa</i>	0.43			
<i>Pseudomonas species</i>			0.54	1.07
<i>Saccharomyces cerevisiae</i>	0.56	0.63		
<b>Anaerobic growth</b>				
<i>Moraxella thermacetica</i>	0.11			
<i>Escherichia coli</i>	0.13			
<i>Klebsiella pneumoniae</i>	0.12			
<i>Saccharomyces cerevisiae</i>	0.12			

Table 5.1 Growth yield ( $Y_{\text{carbon}}$ ) on minimal medium plus various carbon and energy sources

organic acids. Animal fats and plant oils may also be incorporated into some media, often as supplements to the main carbon source.

#### MOLASSES

Pure glucose and sucrose are rarely used for industrial-scale fermentations, primarily due to cost. Molasses, a byproduct of cane and beet sugar production, is a cheaper and more usual source of sucrose. This material is the residue remaining after most of the sucrose has been crystallized from the plant extract. It is a dark coloured viscous syrup containing 50–60% (w/v) carbohydrates, primarily sucrose, with 2% (w/v) nitrogenous substances, along with some vitamins and minerals. Overall composition varies depending upon the plant source, the location of the crop, the climatic conditions under which it was grown and the factory where it was processed. The carbohydrate concentration may be reduced during storage by contaminating microorganisms. A similar product, hydrol molasses, can also be used. This byproduct of maize starch processing primarily contains glucose.

#### MALT EXTRACT

Aqueous extracts of malted barley can be concentrated to form syrops that are particularly useful carbon sources for the cultivation of filamentous fungi, yeasts and actinomycetes. Extract preparation is essentially the same as for malt wort production in beer brewing (Chapter 12). The composition of malt extracts varies to

some extent, but they usually contain approximately 90% carbohydrate, on a dry weight basis. This comprises 20% hexoses (glucose and small amounts of fructose), 55% disaccharides (mainly maltose and traces of sucrose), along with 10% maltotriose, a trisaccharide. In addition, these products contain a range of branched and unbranched dextrans (15–20%), which may or may not be metabolized, depending upon the microorganism. Malt extracts also contain some vitamins and approximately 5% nitrogenous substances, proteins, peptides and amino acids.

Sterilization of media containing malt extract must be carefully controlled to prevent over-heating. The constituent reducing sugars and amino acids are prone to generating Maillard reaction products when heated at low pH. These are brown condensation products resulting from the reaction of amino groups of amines, amino acids and proteins with the carbonyl groups of reducing sugars, ketones and aldehydes. Not only does this cause colour change, but it also results in loss of fermentable materials and some reaction products may inhibit microbial growth.

#### STARCH AND DEXTRINS

These polysaccharides are not as readily utilized as monosaccharides and disaccharides, but can be directly metabolized by amylase-producing microorganisms, particularly filamentous fungi. Their extracellular enzymes hydrolyse the substrate to a mixture of glucose, maltose or maltotriose to produce a sugar spectrum similar to that found in many malt extracts.

Maize starch is most widely used, but it may also be obtained from other cereal and root crops. To allow use in a wider range of fermentations, the starch is usually converted into sugar syrup, containing mostly glucose. It is first gelatinized and then hydrolysed by dilute acids or amylolytic enzymes, often microbial glucoamylases that operate at elevated temperatures.

#### SULPHITE WASTE LIQUOR

Sugar containing wastes derived from the paper pulping industry are primarily used for the cultivation of yeasts. Waste liquors from coniferous trees contain 2-3% (w/v) sugar, which is a mixture of hexoses (80%) and pentoses (20%). Hexoses include glucose, mannose and galactose, whereas the pentose sugars are mostly xylose and arabinose. Those liquors derived from deciduous trees contain mainly pentoses. Usually the liquor requires processing before use as it contains sulphur dioxide. The low pH is adjusted with calcium hydroxide or calcium carbonate, and these liquors are supplemented with sources of nitrogen and phosphorus.

#### CELLULOSE

Cellulose is predominantly found as lignocellulose in plant cell walls, which is composed of three polymers: cellulose, hemicellulose and lignin (see Chapter 10, Bioconversion of lignocellulose). Lignocellulose is available from agricultural, forestry, industrial and domestic wastes. Relatively few microorganisms can utilize it directly, as it is difficult to hydrolyse. The cellulose component is in part crystalline, encrusted with lignin, and provides little surface area for enzyme attack. At present it is mainly used in solid-substrate fermentations to produce various mushrooms (see Chapter 14). However, it is potentially a very valuable renewable source of fermentable sugars once hydrolysed, particularly in the bioconversion to ethanol for fuel use (see Chapter 10).

#### WHEY

Whey is an aqueous byproduct of the dairy industry. The annual worldwide production is over 80 million tonnes, containing over 1 million tonnes of lactose and 0.2 million tonnes of milk protein. This material is expensive to store and transport. Therefore, lactose concentrates are often prepared for later fermentation by evaporation of the whey, following removal of milk proteins for use as food supplements. Lactose is generally less useful as a

fermentation feedstock than sucrose, as it is metabolized by fewer organisms. *S. cerevisiae*, for example, does not ferment lactose. This disaccharide was formerly used extensively in penicillin fermentations and it is still employed for producing ethanol, single cell protein, lactic acid, xanthan gum, vitamin B<sub>12</sub> and gibberellic acid.

#### ALKANES AND ALCOHOLS

*n*-Alkanes of chain length C<sub>10</sub>-C<sub>20</sub> are readily metabolized by certain microorganisms. Mixtures, rather than a single compound, are usually most suitable for microbial fermentations. However, their industrial use is dependent upon the prevailing price of petroleum. Methane is utilized as a carbon source by a few microorganisms, but its conversion product methanol is often preferred for industrial fermentations as it presents fewer technical problems. High purity methanol is readily obtained and it is completely miscible with water. Methanol has a high per cent carbon content and is relatively cheap, although only a limited number of organisms will metabolize it. Also, unlike many other carbon sources, only low concentrations, 0.1-1% (v/v), are tolerated by microorganisms, higher levels being toxic. During fermentations on methanol, the oxygen demand and heat of fermentation are high, but this is even more problematic when growing on alkanes. Several companies used methanol in microbial protein production in the 1970s and early 1980s, but these processes are currently uneconomic (see Chapter 14, Microbial biomass).

Ethanol is less toxic than methanol and is used as a sole or cosubstrate by many microorganisms, but it is too expensive for general use as a carbon source. However, its biotransformation to acetic acid by acetic acid bacteria remains a major fermentation process (see Chapter 12, Vinegar manufacture).

#### FATS AND OILS

Hard animal fats that are mostly composed of glycerides of palmitic and stearic acids are rarely used in fermentations. However, plant oils (primarily from cotton seed, linseed, maize, olive, palm, rape seed and soya) and occasionally fish oil, may be used as the primary or supplementary carbon source, especially in antibiotic production. Plant oils are mostly composed of oleic and linoleic acids, but linseed and soya oil also have a substantial amount of linolenic acid. The oils contain more energy per unit weight than carbohydrates. In

tion, the carbohydrates occupy a greater volume, because they are usually prepared as aqueous solutions of concentrations no greater than 50% (w/w). Consequently, oils can be particularly useful in fed-batch operations, as less spare capacity is needed to accommodate further additions of the carbon source.

### Nitrogen sources

Most industrial microbes can utilize both inorganic and organic nitrogen sources. Inorganic nitrogen may be supplied as ammonium salts, often ammonium sulphate and diammonium hydrogen phosphate, or ammonia. Ammonia can also be used to adjust the pH of the fermentation. Organic nitrogen sources include amino acids, proteins and urea. Nitrogen is often supplied in crude forms that are essentially byproducts of other industries, such as corn steep liquor, yeast extracts, peptones and soya meal. Purified amino acids are used only in special situations, usually as precursors for specific products.

### CORN STEEP LIQUOR

Corn steep liquor is a byproduct of starch extraction from maize and its first use in fermentations was for penicillin production in the 1940s. The exact composition of the liquor varies depending on the quality of the maize and the processing conditions. Concentrated extracts generally contain about 4% (w/v) nitrogen, including a wide range of amino acids, along with vitamins and minerals. Any residual sugars are usually converted to lactic acid (9–20%, w/v) by contaminating bacteria. Corn steep liquor can sometimes be replaced by similar liquors, such as those derived from potato starch production.

### YEAST EXTRACTS

Yeast extracts may be produced from waste baker's and brewer's yeast, or other strains of *S. cerevisiae*. Alternate sources are *Kluyveromyces marxianus* (formerly classified as *K. fragilis*) grown on whey and *Candida utilis* cultivated using ethanol, or wastes from wood and paper processing. Those extracts used in the formulation of fermentation media are normally salt-free concentrates of soluble components of hydrolysed yeast cells. Yeast extracts with sodium chloride concentrations greater than 0.05% (w/v) cannot be used in fermentation processes due to potential corrosion problems.

Yeast cell hydrolysis is often achieved by autolysis, using the cell's endogenous enzymes, usually without the need for additional hydrolytic enzymes. Autolysis can be initiated by temperature or osmotic shock, causing cells to die but without inactivating their enzymes. Temperature and pH are controlled throughout to ensure an optimal and standardized autolysis process. Temperature control is particularly important to prevent loss of vitamins. Autolysis is performed at 50–55°C for several hours before the temperature is raised to 75°C to inactivate the enzymes. Finally, the cells are disrupted by plasmolysis or mechanical disruption. Cell wall materials and other debris are removed by filtration or centrifugation and the resultant extract is rapidly concentrated. Extracts are available as liquids containing 50–65% solids, viscous pastes or dry powders. They contain amino acids, peptides, water-soluble vitamins (Table 5.2) and some glucose, derived from the yeast storage carbohydrates (trehalose and glycogen).

### PEPTONES

Peptones are usually too expensive for large-scale industrial fermentations. They are prepared by acid or enzyme hydrolysis of high protein materials: meat, casein, gelatin, keratin, peanuts, soy meal, cotton seeds, etc. Their amino acid compositions vary depending upon the original protein source. For example, gelatin-derived peptones are rich in proline and hydroxyproline, but are almost devoid of sulphur-containing amino acids; whereas keratin peptone is rich in both proline and cystine, but lacks lysine. Peptones from plant

Table 5.2 Protein and vitamin composition of yeast extract

Total proteins, peptides & amino acids (% w/v)	73–75
free amino acids	35–40
peptides less than 600 Da	10–15
material above 600 Da	20–30
Vitamins (µg/g)	
thiamin	30
riboflavin	120
niacin	700
pyridoxine	20
folic acid	30
calcium pantothenate	300
biotin	2.5

Note: mineral content varies with the processing steps used.

sources invariably contain relatively large quantities of carbohydrates.

#### SOYA BEAN MEAL

Residues remaining after soya beans have been processed to extract the bulk of their oil are composed of 50% protein, 8% non-protein nitrogenous compounds, 30% carbohydrates and 1% oil. This residual soya meal is often used in antibiotic fermentations because the components are only slowly metabolized, thereby eliminating the possibility of repression of product formation.

#### Water

All fermentation processes, except solid-substrate fermentations, require vast quantities of water. In many cases it also provides trace mineral elements. Not only is water a major component of all media, but it is important for ancillary equipment and cleaning. A reliable source of large quantities of clean water, of consistent composition, is therefore essential. Before use, removal of suspended solids, colloids and microorganisms is usually required. When the water supply is 'hard', it is treated to remove salts such as calcium carbonate. Iron and chlorine may also require removal. For some fermentations, notably plant and animal cell culture, the water must be highly purified.

Water is becoming increasingly expensive, necessitating its recycle/reusage wherever possible. This minimizes water costs and reduces the volume requiring waste-water treatment.

#### Minerals

Normally, sufficient quantities of cobalt, copper, iron, manganese, molybdenum, and zinc are present in the water supplies, and as impurities in other media ingredients. For example, corn steep liquor contains a wide range of minerals that will usually satisfy the minor and trace mineral needs. Occasionally, levels of calcium, magnesium, phosphorus, potassium, sulphur and chloride ions are too low to fulfil requirements and these may be added as specific salts.

#### Vitamins and growth factors

Many bacteria can synthesize all necessary vitamins from basic elements. For other bacteria, filamentous

fungi and yeasts, they must be added as supplements to the fermentation medium. Most natural carbon and nitrogen sources also contain at least some of the required vitamins as minor contaminants. Other necessary growth factors, amino acids, nucleotides, fatty acids and sterols, are added either in pure form or, for economic reasons, as less expensive plant and animal extracts.

#### Precursors

Some fermentations must be supplemented with specific precursors, notably for secondary metabolite production. When required, they are often added in controlled quantities and in a relatively pure form. Examples include phenylacetic acid or phenylacetamide added as side-chain precursors in penicillin production. D-threonine is used as a precursor in L-isoleucine production by Serratia marsecens, and anthranilic acid additions are made to fermentations of the yeast Hansenula anomala during L-tryptophan production.

#### Inducers and elicitors

If product formation is dependent upon the presence of a specific inducer compound or a structural analogue, it must be incorporated into the culture medium or added at a specific point during the fermentation. In plant cell culture the production of secondary metabolites, such as flavonoids and terpenoids, can be triggered by adding elicitors. These may be isolated from various microorganisms, particularly plant pathogens (see Chapter 17).

Inducers are often necessary in fermentations of genetically modified microorganisms (GMMs). This is because the growth of GMMs can be impaired when the cloned genes are 'switched on', due to the very high levels of their transcription and translation. Consequently, inducible systems for the cloned genes are incorporated that allow initial maximization of growth to establish high biomass density, whereupon the cloned gene can then be 'switched on' by the addition of the specific chemical inducer.

#### Inhibitors

Inhibitors are used to redirect metabolism towards the target product and reduce formation of other metabolic intermediates; others halt a pathway at a certain point to prevent further metabolism of the target product. An ex-

Example of an inhibitor specifically employed to redirect metabolism is sodium bisulphite, which is used in the production of glycerol by *S. cerevisiae* (see Chapter 10).

Some GMMs contain plasmids bearing an antibiotic resistance gene, as well as the heterologous gene(s). The incorporation of this antibiotic into the medium used for the production of the heterologous product selectively inhibits any plasmid-free cells that may arise.

### Cell permeability modifiers

These compounds increase cell permeability by modifying cell walls and/or membranes, promoting the release of intracellular products into the fermentation medium. Compounds used for this purpose include penicillins and surfactants. They are frequently added to amino acid fermentations, including processes for producing L-glutamic acid using members of the genera *Corynebacterium* and *Brevibacterium* (see Chapter 10).

### Oxygen

Depending on the amount of oxygen required by the organism, it may be supplied in the form of air containing about 21% (v/v) oxygen, or occasionally as pure oxygen when requirements are particularly high. The organism's oxygen requirements may vary widely depending upon the carbon source. For most fermentations the air or oxygen supply is filter sterilized prior to being injected into the fermenter.

### Antifoams

Antifoams are necessary to reduce foam formation during fermentation. Foaming is largely due to media proteins that become attached to the air-broth interface where they denature to form a stable foam. If uncontrolled the foam may block air filters, resulting in the loss of aseptic conditions; the fermenter becomes contaminated and microorganisms are released into the environment. Of possibly most importance is the need to allow 'freeboard' in fermenters to provide space for the foam generated. If foaming is minimized, then throughputs can be increased.

There are three possible approaches to controlling foam production: modification of medium composition, use of mechanical foam breakers and addition of chemical antifoams. Chemical antifoams are surface-active agents which reduce the surface tension that binds

the foam together. The ideal antifoam should have the following properties:

- 1 readily and rapidly dispersed with rapid action;
- 2 high activity at low concentrations;
- 3 prolonged action;
- 4 non toxic to fermentation microorganisms, humans or animals;
- 5 low cost;
- 6 thermostability; and
- 7 compatibility with other media components and the process, i.e. having no effect on oxygen transfer rates or downstream processing operations.

Natural antifoams include plant oils (e.g. from soya, sunflower and rapeseed), deodorized fish oil, mineral oils and tallow. The synthetic antifoams are mostly silicon oils, poly alcohols and alkylated glycols. Some of these may adversely affect downstream processing steps, especially membrane filtration.

### Animal cell culture media

Animal cell culture media are normally based on complex basal media, such as Eagle's cell culture medium, which contains glucose, mineral salts, vitamins and amino acids. For mammalian cells a serum is usually added, such as fetal calf serum, calf serum, newborn calf serum or horse serum. Sera provide a source of essential growth factors, including initiation and attachment factors, and binding proteins. They also supply hormones, trace elements and protease inhibitors.

The highly complex composition of sera makes substitution with lower cost ingredients very difficult. Sterilization of formulated animal culture media and media constituents is also more problematic as many components are thermolabile, requiring filter sterilization. Normally, sera constitute 5-10% (v/v) of the medium, but attempts have been made to reduce and ultimately eliminate its use. This is necessary due to its high cost and the fact that it is a potential source of prions and viruses. In some circumstances levels have now been lowered to 1-2% (v/v) and some cell lines have been developed that grow in serum-free media.

### Plant cell culture media

In contrast to most animal cell culture media, those used for plant cell culture are usually chemically defined. They contain an organic carbon source (as most plant cells are grown heterotrophically), a nitrogen source,