Fermentation media

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 .

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 C4H7O2N, 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 C3.72H6.11 O1.95 N0.61 S 0.017 P0.035 and K0.056. 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.

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 .

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. 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. 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, 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*ATP (yield of cells per mole of ATP generated during growth) can be calculated if the metabolism of the organism has been fully elucidated.

Carbohydrates are traditional carbon and energy sources for microbial fermentations, although other sources may be used, such as alcohols, alkanes and 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 industrialscale 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 syrups 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 . The composition of malt extracts varies to some extent, but they usually contain approximately90% carbohydrate, on a dry weight basis. This comprises20% 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 dextrins (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 . 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 . However, it is potentially a very valuable renewable source of fermentable sugars once hydrolysed, particularly in the bioconversion to ethanol for fuel use .

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 B12 and gibberellic acid.

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 addition, 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 at50–55°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, watersoluble 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, gelatinderived 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 sources invariably contain relatively large quantities of carbohydrates.

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 600Da	10–15
material above 600Da	20–30
Vitamins (mg/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.

SOYA BEAN MEAL:

Residues remaining after soya beans have been processed to extract the bulk of their oil are composed of50% 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. dthreonine is used as a precursor in l-isoleucine production by *Serratia marsescens,* and anthranillic 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 .

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 example of an inhibitor specifically employed to redirect metabolism is sodium bisulphite, which is used in the production of glycerol by *S. cerevisiae* . 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 lglutamic acid using members of the genera *Corynebacterium* and *Brevibacterium* .

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 surfaceactive 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 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, mineral salts and growth hormones. Sucrose is frequently incorporated as the carbon source, particularly for secondary metabolite production, but glucose, fructose, maltose and even lactose have been used. Nitrate is the usual

nitrogen source, often supplemented with ammonium salts. However, some species may require organic nitrogen, normally in the form of amino acids. The combination and concentration of plant hormones provided depend upon the specific fermentation. Auxins are usually supplied, along with cytokinins to promote cell division. A two-phase culture has often proved to be useful in increasing productivity, particularly for producing secondary metabolites such as shikonin . The first phase uses a medium optimized for growth, the second promotes product formation.

Culture maintenance media:

These media are used for the storage and subculturing of key industrial strains. They are designed to retain good cell viability and minimize the possible development of genetic variation. In particular, they must reduce the production of toxic metabolites that can have strain-destabilizing effects. If strains are naturally unstable, they should be maintained on media selective for the specific characteristic that must be retained.