MICROBIAL CELL CULTURE

Horst W. Doelle
MIRCEN-Biotechnology Brisbane and Pacific Regional Network, Brisbane, Australia

Keywords: substrate, Nutrition, product formation, nutrients, Macronutrients, chemoheterotrophs

Contents

Summary

Microbial cells have two main commercial applications. Firstly, the cells can be used as a source of protein, primarily for animal feed, which require maximal growth

conditions. Secondly, they can be used to carry out biological conversions and thus lead to organic chemical production. These biological conversions and or microbial transformations can be accomplished with growing cells, non-growing cells, immobilized cells, spores or even dried cells, which requires media design and flexibility in cultivation systems. The design of growth and production media includes constraints such as cost, availability of raw materials, requirement of specific carbon, nitrogen and other nutritional sources.

The use of microbial cell cultures for commercial purposes is always aimed at maximizing three factors:

(a) Yield of product per gram of substrate;
(b) Concentration of the product;
(c) Rate of product formation.

All three aspects are basically concerned with the adjustment of metabolic regulation in the organism, whereby metabolism means that all of the available carbon is converted into biomass and/or product, requiring stoichiometric evaluations.

Since microorganisms, apart from their constant genotype, are amazingly flexible in their ability to alter their chemical composition and metabolism in response to environmental changes, flexibility in medium design, optimization, and the type of cultivation system used, is necessary. It is the challenge of microbiologists and microbial technologists to develop the right combination of medium and cultivation system from those outlined in this article. If one wants to develop a technology of a process one has to know the catalyst first. The catalyst is the appropriate microorganism and its suitability for a process that falls under microbial process development.

1. Introduction

In terms of the total biomass of our planet, microorganisms are equal to the animal kingdom (including humans). The question was thus raised whether mankind has taken, or is taking, full advantage of this almost untapped natural resource. Microorganisms are frequently referred to as the cause of disease in human beings, animals and plants, and only slowly are we recognizing that many more types are beneficial than harmful to higher forms of life. The reason for this increasing awareness over the last decade is the realization that biological systems may be utilized for many new purposes in addition to food production. It is the biological sciences, which are expected to provide important potentialities for development in the forthcoming millennium.

2. Nutrition

All substances in the environment, which can be used by the cell for catabolism and biosynthesis are called nutrients. A culture medium must therefore contain all necessary nutrients in quantities appropriate to the specific requirements of the microorganisms for which it is designed. However, microorganisms are extraordinarily diverse in their specific physiological properties, and correspondingly in their nutrient requirements. Literally thousands of different media have been proposed for their cultivation, and in

the description of these media the reasons for the presence of the various components are often not clearly stated. Nevertheless, the design of a culture medium can and should be based on scientific principles: the principles of nutrition.

The chemical composition of the cell, broadly constant throughout the living world, indicates the major material requirements for growth. Water accounts for some 80 to 90 percent of the total weight of cells and is always therefore the major essential nutrient in quantitative terms. In addition to hydrogen and oxygen, the solid matter of cells contains carbon, nitrogen, phosphorous and sulfur, in order of decreasing abundance. These six elements account for about 95 percent of the cellular dry weight.

The nutrients can be divided into two major classes:

1. Necessary nutrients, without which the cell can not grow;
2. Useful, but dispensable nutrients, which are used if present, but are not essential.

Some nutrients are the building blocks from which the cell makes macromolecules and other structures, while other nutrients serve only as the energy source without being incorporated directly into the cellular material. Sometimes a nutrient can play both roles.

The required substances can therefore be divided into two groups - macronutrients and micronutrients - depending on whether they are required in large or small amounts.

2.1. Macronutrients

2.1.1. Carbon Source

The carbon source is obviously one of the most important nutrients in the growth of microorganisms. The element carbon is the most abundant element and represents approximately 50 percent of the biomass. If it is a limiting factor, the total biomass X is proportional to the initial concentration of the organic source of carbon, which gives the yield constant for the substrate and organism:

$$Y_s = X$$

It is therefore possible to calculate the minimum quantity of a carbon substrate to obtain a specific yield of biomass.

2.1.2. Nitrogen Source

Nitrogen, which is needed for amino acids, purine and pyrimidine biosynthesis, can be obtained by microorganisms from either inorganic or organic forms. The most favorable inorganic nitrogen sources are nitrate and ammonia. Although ammonia has the same oxidation state as an amino group, its assimilation into amino acids still requires expenditure of energy. The most common way is the direct introduction of an amino group in exchange for a keto group:

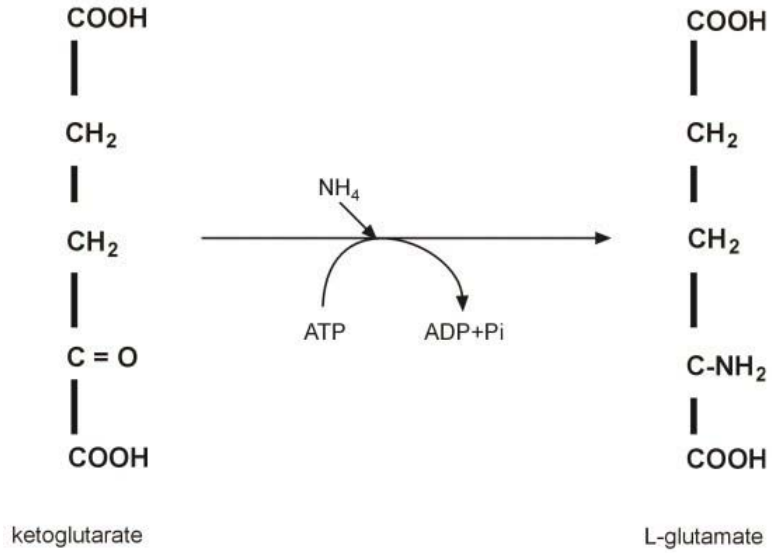

Figure 1: Incorporation of ammonia-N into a keto acid

Once the amino acid has been incorporated into glutamate, transaminases transfer the amino group into the appropriate carbon skeleton.

When nitrate is used as a nitrogen source, it has to be reduced to ammonia first, a process called "assimilatory nitrate reduction", which involves the enzymes nitrate reductase and nitrite reductase. A third possibility is the utilization of nitrogen gas (N_2) as a source of nitrogen, a process called "nitrogen fixation". This process is a property of only certain bacteria and cyanobacteria. In the fixation process, dinitrogen is reduced to ammonia, which then can be used as mentioned earlier.

2.1.3. Sulfur Source

Inorganic sulfate is absolutely necessary for growth to synthesize the sulfur-containing amino acids cysteine and methionine as well as the vitamins thiamine, biotin and lipoic acid. The assimilation of sulfate first involves its activation by a reaction with ATP in two steps to form phosphoadenosine phosphosulfate (PAPS). Subsequently, the sulfate radical attached to PAPS is reduced to sulfite (SO_3^{2-}), which is further reduced to hydrogen sulfide (H_2S). The incorporation of the sulfur into organic sulfur compounds always occurs via serine.

2.1.4. Phosphorous Source

Phosphorous occurs in nature in the form of organic and inorganic phosphates and is utilized by microorganisms primarily to synthesize phospholipids and nucleic acids. Thus, all microorganisms utilize inorganic phosphate for growth. Organic phosphate compounds in nature are utilized as phosphate sources through the action of phosphatases, which are enzymes hydrolyzing the organic phosphate ester.

2.1.5. Others

A variety of other minerals are required for growth, such as potassium, magnesium, calcium, and in some cases silicon. Of those, magnesium is an essential nutrient as it functions to stabilize ribosomes, cell membranes and nucleic acids. Magnesium is also required for the activity of many enzymes especially those involving phosphate transfer. Gram-positive bacteria require about ten times more magnesium than gram-negative bacteria. Without magnesium, there is no growth possible.

Calcium ions play a key role in the heat stability of bacterial spores and may also be involved in the stability of the cell wall. Calcium, however, can't replace magnesium. Potassium is universally required for the activation of some enzymes involved in protein biosynthesis.

Sodium requirement reflects only the environment. For example, seawater has a high sodium content and thus marine microorganisms generally require sodium for growth.

2.2. Micronutrients

2.2.1. Tracer elements

The requirements for tracer elements are difficult to determine, since most macronutrients contain enough tracer elements to satisfy the demand. The tracer elements commonly required by most microorganisms are zinc, copper, manganese and molybdenum. These metals function in enzymes or coenzymes.

2.2.2. Iron

Iron is a rather special case, as it is required in significant amounts, although not at a level of macronutrients. Since iron is normally present in the environment in a very insoluble form, organisms must have special mechanisms for obtaining iron from their habitats. Iron has two oxidation states, ferrous (Fe^{2+}) and ferric (Fe^{3+}), with the ferrous compounds generally more soluble. Iron forms complexes with a wide variety of organic compounds, specifically with metals called chelators. These chelators play a special role in iron transport. Many microorganisms produce specific iron-binding organic compounds called ironophores, which solubilize ferric ions and transport them into the cell. Some of these ironophores are also referred to as siderochromes or ferrichromes. Mostly, these organic compounds are derivatives of hydroxamic acid or phenolic acids. In culture media, iron is rendered available by providing it in chelated form with a synthetic chelating agent EDTA (ethylenediaminotriacetic acid) or NTAA (nitrolotriacetic acid). A concentration of 10 $\mu g ml^{-1}$ would be in excess for virtually any microbial culture.

2.3. Growth Factors

Growth factors are specific organic compounds that are required in very small amounts and are unable to be synthesized by the cell. Substances frequently serving as growth factors are vitamins, amino acids, purines, and pyrimidines. In practice, any deficiency in biosynthesis or requirement for growth is compensated by the addition of yeast extract or peptone.

These growth factor requirements have been widely employed for the examination of foods, pharmaceuticals and other preparations. Such " microbiological assays" have the virtues of specificity, sensitivity, and simplicity. To perform the assay, a culture medium is used in which all substances needed by the microorganism for growth are supplied, with the exception of the substance to be assayed. The growth factor is then added to the medium at some low concentration. Under these conditions, the amount of growth obtained after incubation is proportional to the concentration of the limiting growth factor:

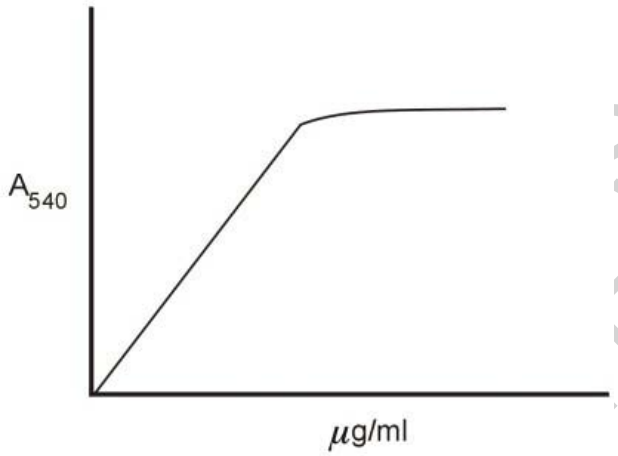

Figure 2: Microbiological Assay

Growth factor requirements are greatest under anaerobic growth conditions and the least under aerobic conditions.

2.4. Medium Composition

In constructing a culture medium for any microorganism, the primary goal is to provide a balanced mixture of the required nutrients at concentrations that will permit good growth. It might seem at first sight reasonable to make the medium as rich as possible by providing all nutrients in great excess. However, this approach is not a wise one. In the first place, many nutrients become growth inhibitory or toxic as the concentration is raised. This is true of many organic substrates, such as salts of fatty acids and even of sugars. Some inorganic constituents may also become inhibitory if supplied in excess. Second, even if growth can occur in a concentrated medium, the metabolic activities of the growing microbial population will eventually change the nature of the environment to the point where it becomes highly unfavorable and the population becomes physiologically abnormal or dies.

The rational point of departure for the preparation of media is to compound a mineral base, which provides all these nutrients that can be supplied to any organism in inorganic form. This base can then be supplemented as required with a carbon source, an energy source, a nitrogen source, and any required growth factor.

A medium composed entirely of chemically defined nutrients is termed a "synthetic" medium. One that contains ingredients of unknown chemical composition is termed a "complex" medium.

In microbiology, every medium is finally sterilized before inoculation with the one specific microbial strain under investigation. It is not wise to sterilize the mineral base medium containing the carbon source, particularly if sugars are involved. Sugars caramelize in the presence of inorganic salts and thus only become partly available for microbial utilization. Carbon sources and mineral base solutions should be sterilized separately and mixed aseptically prior to inoculation.

In order to encompass the variety of nutritional patterns known to exist amongst bacteria, the following nutritional terminology has been introduced:

- Autotroph: A microorganism that is able to use carbon dioxide as sole carbon source for growth (cell carbon);
- Heterotroph: A microorganism that requires carbon sources more reduced than carbon dioxide for growth (cell carbon);
- Photolithotroph: A microorganism that derives its energy from light and uses inorganic compounds as electron donor (mostly photoautotrophs);
- Photoorganotroph: A microorganism that derives its energy from light and uses organic compounds as electron donor (mostly photoheterotrophs);
- Chemolithotrophs: A microorganism that derives its energy from biochemical reactions and uses inorganic compounds as electron donor (mostly chemo-autotrophs);
- Chemoorganotrophs: A microorganism that derives its energy from biochemical reactions and uses organic compounds as electron donor (mostly chemoheterotrophs).

In order to take into account the requirement for growth factors an additional pair of terms, prototrophy and auxotrophy, are sometimes employed. A prototroph can derive all carbon requirements from the principal carbon source, whereas an auxotroph requires in addition to the principal carbon source one or more organic nutrients.

3. Growth

In order to follow the course of growth, it is necessary to make quantitative measurements. As a matter of convenience, the properties measured are usually cell mass or cell number.

3.1. Measurement

3.1.1. Dry Weight

The only direct way to measure cell mass is to determine the dry weight of cell material in a fixed volume of culture by removing the cells from the medium, washing them in water or buffer solutions, drying them typically at 80°C for 24h or at 110°C for 8h, and then weighing them to constancy. Such determinations are time consuming and relatively insensitive. Furthermore, it is difficult to weigh with an accuracy of less than 1 mg, the dry weight of which may still represent as many as 5 billion bacteria.

3.1.2. Optical Measurement

The determination of the amount of light scattered by a suspension of cells is a technique based on the fact that small particles scatter light proportionally, within certain limits, to their concentration. When a beam of light is passed through a suspension of bacteria, the reduction in the amount of light transmitted as a consequence of scattering is thus a measure of the bacterial mass present. Such measurements are usually made in a photometer or spectrophotometer using appropriate wavelengths.

Absorbency is defined as the logarithm of the ratio of light striking the suspension (*Io*) to that transmitted by the suspension (*I*):

$$A = \log \frac{Io}{I}$$

Since scattering is inversely proportional to the fourth power of the wavelength of light being scattered, the sensitivity of the measurements increases sharply if light of shorter wavelength is used. In general, however, the lower limit of sensitivity of the method is reached with bacterial suspensions that contain about 10 million bacteria/ml. Thus the proportionality between absorbency and dry weight is linear only at low values of absorbance (e.g. up to approx. 0.5 absorbency units).

-
-
-

Bibliography

Almanza S., Crooke P. S., Desgranges C., Durand A., Hong K., Lonsane B. K., Malaney G. W., Maratray J., Mitchell D. A., Renaud R., and Tanner R. D.(1992). General Principles of Reactor Design and Operation for SSC. *Solid Substrate Cultivation*, (eds. HW Doelle, DA Mitchell and CA Rolz), pp.115-140 London: Elsevier Applied Science. [In this article the emphasis is given to chemical engineering aspects in the application of solid substrate cultivation systems.]

DaSilva E. J. (1980). Trends in microbial technology for developing countries. *Renewable Resources - A systematic approach*, (ed. E Campos-Lopez), pp. 329-368. London: Academic Press. [This article outlines that microbial technology can help provide solutions depending on the country's own characteristic problems.]

Doelle H. W. (1975). *Bacterial Metabolism*. New York: Academic Press [This book describes the fundamentals of bacterial activities.]

Doelle H. W. (1991). Microbial Process Development. *The Importance of Microbiological Biotechnology for Community and Economic Development*, UNESCO-MIRCEN Regional Training Course, Motupore Island, University of Papua New Guinea, September 1991 [This training course was designed for infrastructure development in the Pacific Region.]

Doelle H. W., Mitchell D. A. and Rolz C. E., eds. (1992). *Solid Substrate Cultivation*, 466 pp. London: Elsevier Applied Science. [This book deals with all aspects of solid-state cultivation and processes of

microorganisms.]

Doelle H. W. (1994). *Microbial Process Development.* Singapore: World Scientific Publishers. [This book deals with the application of microbiological, biochemical and chemical engineering fundamentals in the field.]

Doelle H. W. (1997). Solid state cultivation: basic concept and strategies. *Treatment and Utilization of Agro-Industrial Waste for a Cleaner Environment and Sustainability,* ICRO/UNESCO/MIRCEN/BIOTEC International Regional Training Course, Prince of Songkla University, Hat Yai, Thailand, August 1997. [This training course was specifically designed for the use of various aspects of biotechnology in agro-industries of SEAsia, where solid-state cultivation systems can improve farm income.]

Hamer G. (1997). Theoretical analysis of microbial growth I & II. *Treatment and Utilization of Agro-Industrial Waste for a Cleaner Environment and Sustainability.* ICRO/UNESCO/MIRCEN/ BIOTEC International Regional Training Course, Prince of Songkla University, Hat Yai, Thailand, August 1997. [This article was part of a training course emphasizing the use of bacterial systems in waste management.]

Lonsane B. K., Ghildyal N. P., Budiatman S., and Ramakrishna S. V. (1985). Engineering aspects of solid state fermentation. *Enzyme Microbiological Technology* **7**, 258-265. [This article specifically stresses the engineering aspects in an otherwise microbiological cultivation system.]

Mitchell D. A. (1992). Microbial Basis of Processes. *Solid Substrate Cultivation* (eds. H. W. Doelle, D. A. Mitchell, and C. A. Rolz), pp. 17-28. London: Elsevier Applied Science. [This article outlines the microbial fundamentals of solid substrate cultivation systems.].

Mitchell D. A. and Lonsane B. K. (1992). Definition, characteristics and potential. *Solid Substrate Cultivation* (eds. H. W. Doelle, D. A. Mitchell and C. A. Rolz), pp.1-16. London: Elsevier Applied Science. [This article deals essentially with the definition, characteristics and the biotechnological potential of solid substrate cultivation techniques.]

Pirt S. J. (1975). *Principles of Microbe and Cell Cultivation.* London: Blackwell Scientific Publications. [This book forms the basis for all cell cultivation systems.]

Sahm H., ed. (1993). Biological fundamentals. *Biotechnology* (eds. A. Puhler, G. Reed, H. J. Rehm, P. Stadler), 2nd ed., Vol. 1, 640 pp. Germany: VCH Verlagsgesellschaft Weinheim. [This volume of the multi-volume comprehensive treatise deals in details with the biological background material for the development of biotechnological processes.]

Biographical Sketch

Horst W. Doelle, born in 1932, studied biology at the University of Jena [1950-1954]. He studied for his doctorate at University of Goettingen [1955-1957] on antibiotic production. After receiving his doctorate, he worked in the wine and brewing industry in Germany before taking up an appointment with CSIRO in Australia in 1960. After 4 years wine research, he took up the challenge to build up microbial physiology and fermentation technology at the Department of Microbiology at the University of Queensland in Brisbane. He received his Doctor of Science in 1976 and his Doctor of Science *honoris causa* in 1998. He participated and conducted numerous training courses in developing countries. After 29 years teaching he retired in 1992. His research area was regulation of anaerobic/aerobic metabolism, microbial technology [*Zymomonas* ethanol technology] and socio-economic biotechnology using microorganisms for waste management.