Mixed Cultures in Biotechnology

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J. Gregory Zeikus
Michigan Biotechnology Institute
Lansing, Michigan

Department of Biochemistry
Department of Microbiology and Public Health
Michigan State University
East Lansing, Michigan

Eric A. Johnson
Department of Food Microbiology and Toxicology
University of Wisconsin
Madison, Wisconsin

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Contributors

Lakshmi Bhatnagar Michigan Biotechnology Institute, Lansing, Michigan; Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan. (CHAP. 11)

Linda F. Bisson Department of Viticulture and Enology, University of California, Davis, California. (CHAP. 3)

Nicholas J. E. Dowling Institute for Applied Microbiology, University of Tennessee, Knoxville, Tennessee. (CHAP. 12)

Babu Z. Fathepure Department of Civil & Environmental Engineering, Michigan State University, East Lansing, Michigan. (CHAP. 11)

Henry P. Fleming Food Fermentation Laboratory, U.S. Department of Agriculture, Agriculture Research Service, and Department of Food Science, North Carolina State University, Raleigh, North Carolina. (CHAP. 4)

Stoyan N. Groudev Higher Institute of Mining and Geology, Research and Training Center of Mineral Biotechnology, Sofia (1156), Bulgaria. (CHAP. 13)

C. W. Hesseltine Consultant, Peoria, Illinois. Retired from Department of Agriculture, Agricultural Research Service, Northern Regional Research Center, Peoria, Illinois. (CHAP. 1)

Eric A. Johnson Department of Food Microbiology and Toxicology, University of Wisconsin, Madison, Wisconsin. (CHAP. 6)

Bruce C. Kelley CRA, Roseville, New South Wales, Australia. (CHAP. 13)

Ralph E. Kunkee Department of Viticulture and Enology, University of California, Davis, California. (CHAP. 3)

Marc W. Mittelman Institute for Applied Microbiology, University of Tennessee, Knoxville, Tennessee. (CHAP. 12)

Richard E. Muck U.S. Department of Agriculture, Agricultural Research Service, U.S. Dairy Forage Research Center, Madison, Wisconsin. (CHAP. 7)

Henry J. Peppler Consultant, Whitefish Bay, Wisconsin. Retired from Universal Foods, Milwaukee, Wisconsin. (CHAP. 2)

Badal C. Saha Michigan Biotechnology Institute, Lansing, Michigan; Department of Food Science and Human Nutrition, Michigan State University, East Lansing, Michigan. (CHAP. 9)

Mary Ellen Sanders Consultant, Littleton, Colorado. (CHAP. 5)

Jürgen H. Thiele Gesellschaft für Biotechnologische Forschung mbH, D3300 Braunschweig, Federal Republic of Germany. (CHAP. 10)

Olli H. Tuovinen Department of Microbiology, Ohio State University, Columbus, Ohio. (CHAP. 13)

Paul J. Weimer U.S. Department of Agriculture, Agriculture Research Service, U.S. Dairy Forage Research Center and Department of Bacteriology, University of Wisconsin, Madison, Wisconsin. (CHAP. 8)

David C. White Institute for Applied Microbiology, University of Tennessee, Knoxville, Tennessee. (CHAP. 12)

Preface

Mixed-culture biotechnology is integral to many important industrial processes used throughout the world. Also, mixed-culture fermentations, including those used in the production of food and beverages, have been in use since antiquity. Moreover, new fermentation processes, such as those used in the manufacture of fine chemicals and for the degradation of xenobiotics and pesticides, are being developed in response to new opportunities and demands. Mixed cultures, in fact, offer the advantages of combining in a single process many genetic traits from a variety of organisms—and without employing recombinant DNA. This permits the development and application of complex processes which would be difficult or impossible with pure cultures.

The biological interactions involved in most mixed-culture processes are complex and not fully understood. In this book experts on diverse types of mixed cultures identify common principles shared by various types of fermentations. Also, they emphasize the fundamental roles and interactions of the microorganisms involved. The book is intended to convey principles for improving present-day fermentation processes and for facilitating the development of new biotechnology.

We wish to express our appreciation to the contributors for their care and expertise in preparing their chapters, to Arnold L. Demain for inspiring this project, and to the McGraw-Hill editorial staff.

Eric A. Johnson
J. Gregory Zeikus

ABOUT THE EDITORS

J. Gregory Zeikus is president of the Michigan Biotechnology Institute in Lansing, Michigan and distinguished professor in the Department of Biochemistry and in the Department of Microbiology and Public Health at Michigan State University.

Eric A. Johnson is associate professor in the Department of Food Microbiology and Toxicology at the University of Wisconsin, Madison.

Contents

Chapter

1

Mixed-Culture Fermentations: An Introduction to Oriental Food Fermentations

C. W. Hesseltine

Agricultural Research Service
U. S. Department of Agriculture
Northern Regional Research Center
Peoria, Illinois

Introduction

Mixed-culture fermentations are those in which the inoculum always consists of two or more organisms. As opposed to this, pure cultures consist of only one strain of microorganism growing by itself and excludes inocula in which two or more strains of the same species are used. Mixed cultures can consist of known species to the exclusion of all others, or they may be composed of mixtures of unknown species. The mixed cultures may be all of one microbial group—all bacteria—or they may consist of a mixture of organisms of fungi and bacteria or fungi and yeasts or other combinations in which the components are quite unrelated. All of these combinations are encountered in oriental food fermentations.

A few special situations occur in nature where a single microorganism may be growing alone. The interior of stems, roots, leaves, and seeds are normally sterile. When these parts become infected with a pathogen, this organism may grow in pure culture in the host. Likewise, in extreme environmental conditions such as hot springs or very alkaline environments, a single microorganism may be responsible for the fermentation of a substrate.

The earliest studies of microorganisms were those made on mixed cultures by van Leeuwenhoek (1684). Micheli, working with fungi in 1718, reported his observations of the germination of mold spores on cut surfaces of melons and quinces. Brefield in 1875 obtained pure cultures of fungi, and Koch in 1878 obtained pure cultures of pathogenic bacteria. The objective of both Brefield's and Koch's studies was to identify pathogenic microorganisms. They wanted to prove what organism was actually responsible for a particular disease. Thus, part of Koch's fame rests on his discovery of the cause of tuberculosis. A second interest in pure cultures was the use of a particular microorganism to produce an organic compound by fermentation. For a more detailed discussion of the history of pure cultures, see Chap. 1 in Quayle and Bull (1982) and Buller (1915). The history of the discovery of methods for pure-culture techniques is important because without these methods, the classification of the components of mixed cultures could not have occurred.

An early paper on mixed-culture food fermentation was an address by Macfadyen at the Institute of Brewing, London, in 1903 entitled, "The Symbiotic Fermentations," in which he referred to mixed-culture fermentations as "mixed infections." Probably this expression reflected his being a member of the Jenner Institute of Preventive Medicine. About half of his lecture was devoted to mixed-culture fermentations of the Orient. Among those described were Chinese yeast, *koji*, Tonkin yeast, and *ragi*. In these preparations fungi and yeast were present, and in 1902 they were used commercially to produce alcohol in France.

Mixed cultures are the rule in nature; therefore, one would expect this condition to be the rule in fermented foods of relatively ancient origin. Soil is a mixed-organism environment with protozoa, bacteria, fungi, and algae growing in various numbers and kinds, depending upon the nutrients available, the temperature, and the pH of the soil. Soil microorganisms relate to each other, some as parasites on others, some forming substances essential to others for growth, and some which have no effect on each other. Four special mixed-culture situations exist in nature. The first is the lichens in which fungi and algae grow in such close relationship that groups of cells of the two organisms serve as propagules for reproduction. They are important because they can grow under extreme climatic conditions to form soil from the breakdown of rock.

A second example occurs in a mixed-plant *Rhizobium* interaction in which the bacterium fixes nitrogen in nodules formed on the plant's roots. Thirdly, if we did not have mixed cultures, cellulose might never be completely broken down to water and CO_2 in nature. If the animal and plant debris could not be degraded for the lack of mixed cultures, life would become impossible. Winogradsky (1949) demonstrated that mixed cultures better ferment cellulose than a single microorganism. Besides cellulose, there are many other complex compounds formed by plants and animals. To prevent these from accumulating, series of microorganisms degrade the compounds in various steps to simpler materials which can be used by other organisms. A fourth important mixed-culture fermentation is the activities of bacteria, protozoa, and fungi in the rumens of ruminant animals. Many of the interactions and growth of microorganisms in these fermentations are also found in fermented foods.

Advantages to Mixed-Culture Fermentations

Mixed-culture fermentations offer a number of advantages over conventional fermentations using a single pure culture.

1. Product yield may be higher. Yogurt is made by the fermentation of milk with *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. Driessen (1981) demonstrated that when these species were grown separately, 24 and 20 mmol of acid was produced; together with the same amount of inoculum, a yield of 74 mmol was obtained. The number of *S. thermophilus* cells increased from 500×10^6 per milliliter to 880×10^6 per milliliter with *L. bulgaricus*.

2. The growth rate may be higher. In mixed culture, one microorganism may produce needed growth factors or produce essential growth compounds such as carbon or nitrogen sources beneficial to a second microorganism. It may alter the pH of the medium, thereby improving the activity of one or more enzymes. Even the temperature may be elevated and promote growth of a second microbe.

3. Mixed cultures offer more protection against contamination. In nature pure cultures rarely exist and consequently are less likely to become contaminated. Typically one organism will attack the primary organic material and the by-products are in turn attacked by secondary organisms. Koji mold (several strains used together) typically produces amylases and proteases which break down starch in rice and protein in soybeans. In the *miso* and *shoyu* fermentations, these compounds are in turn acted on by lactic acid bacteria and yeast. In such processes, especially with a heavy inoculum of selected strains, con-

tamination does not occur even when the fermentations are carried out in open pans or tanks allowing air contamination to occur.

4. In some mixed cultures a remarkably stable association of microorganisms may occur. Even when a mixture of cultures is prepared by untrained individuals working under atrociously dirty conditions, such as in *ragi*, mixtures of the same fungi, yeasts, and bacteria remain together even after years of subculture. Probably the steps in making the starter were established by trial and error and the process was handed down from one generation to the next, with the conditions being such that this mixture could compete against all contaminants.

5. Compounds made by a mixture of microorganisms often complement each other and work to the exclusion of unwanted microorganisms. In many of these associations various chemicals are produced that complement each other to the exclusion of undesirable microorganisms. For example, in some food fermentations yeast will produce alcohol and lactic acid bacteria will produce lactic acid and other organic acids and change the environment from aerobic to anaerobic. Inhibiting compounds are thus formed, the pH lowered, and anaerobic conditions are developed excluding most undesirable molds and bacteria.

6. Mixed cultures permit better utilization of the substrate. The substrate for fermented food is always a complex mixture of carbohydrates, proteins, and fats. Mixed cultures possess a wider range of enzymes and are able to attack a greater variety of compounds. Likewise, with proper strain selection they are better able to change or destroy toxic or noxious compounds which may be in the fermentation substrate.

7. Mixed cultures are able to bring about multistep transformations that would be impossible for a single microorganism. An example is the *miso* fermentation where *Aspergillus oryzae* strains are used to make *koji* to convert rice starch to sugar; yeast in turn uses the sugar to make small amounts of alcohol, and bacteria use the sugars to make acid and flavoring substances.

8. Mixed cultures can be maintained indefinitely by unskilled people with a minimum of training. If the environmental conditions can be maintained, i.e., temperature, mass of fermenting substrate, length of fermentation, and kind of substrate, it is easy to maintain a mixed-culture inoculum indefinitely and to carry out repeated successful fermentations.

9. In mixed-culture fermentations phage infections are reduced. In

pure-culture commercial fermentations involving bacteria and actinomycetes, invariably an epidemic of phage infections occurs, and the infection can completely shut down production. Since mixed cultures have a wider genetic base of resistance to phage, failures do not often occur because one strain is wiped out; a second or third phage-resistant strain in the inoculum will take over and continue the fermentation.

10. Simultaneous changes in the substrates may occur. Many different changes in the substrate may occur at the same time, which would not be possible if only one microorganism was used. Harrison (1978) cites an example of a stable enrichment culture in which four types of bacteria were present, one of which used methane; a second used methanol, but not methane; and two bacterial species would not grow on either methane or methanol. It was suggested that the role of the two heterotrophic bacteria was to remove complex products of growth and lysis. Methanol was removed by one autotrophic organism down to the level where it was not inhibiting to the methane-utilizing organism.

11. Mixed-culture fermentations enable the utilization of cheap and impure substrates. In any practical fermentation the cheapest substrate is always used, and this will not be of the highest purity and often will be a mixture of several materials in large quantities. For example, in the production of biomass, a mixed culture is desirable that not only attacks the cellulose but also starch and sugar. Cellulolytic fungi along with starch and sugar-utilizing yeasts would give a more efficient process, producing more biomass in a shorter time.

12. Mixed cultures can provide necessary nutrients for optimal performance. Many microorganisms, such as the cheese bacteria, which might be suitable for production of a fermentation product, require growth factors to achieve optimum growth rates. To add the proper vitamins to production adds complications and expense to the process. Thus, the addition of a symbiotic species which supplies the growth factors is a definite advantage in many types of fermentations.

Harrison (1978) mentioned a specialized advantage in single-cell protein fermentation. When methanol is the substrate, foaming is a large problem. However, when three cultures were used together, foaming did not occur, although it was a problem when each culture was grown separately, and an antifoaming compound had to be added during fermentation.

Disadvantages to Mixed-Culture Fermentations

Mixed-culture fermentations also have some disadvantages.

1. The products formed by mixed-culture fermentation may be more variable than in pure-culture fermentations. In mixed-culture fermentations, products may vary in amount and composition. The importance of the amounts of product produced may depend on what is wanted as an end product. If, on one hand, removal of waste is a problem, variation in amount of product may not be very important. On the other hand, if the objective is to obtain a specific product in maximum yield, then variation in yield becomes very important.
2. Scientific study of mixed cultures is difficult. Obviously it is more difficult to study the fermentation if more than one microorganism is involved. That is why most biochemical studies are conducted as single-culture fermentations because one variable is eliminated.
3. Defining the product and the microorganisms employed becomes more involved in patent and regulatory procedures.
4. Contamination of the fermentation is more difficult to detect and control.
5. When two or three pure cultures are mixed together, it requires more time and space to produce several sets of inocula rather than just one.
6. One of the worst problems in mixed-culture fermentation is the control of the optimum balance among the microorganisms involved. This can, however, be overcome if the behavior of the microorganisms is understood and this information is applied to their control.

The balance of organisms brings up the problem of the storage and maintenance of the cultures. Lyophilization presents difficulties because, in the freeze-drying process, the killing of different strains' cells will be unequal. It is also difficult, if not impossible, to grow a mixed culture from liquid medium in contrast to typical fermentations on solid medium, without the culture undergoing radical shifts in population numbers. According to Harrison (1978), the best way to preserve mixed cultures is to store the whole liquid culture in liquid nitrogen below 80°C. The culture, when removed from the frozen state, should be started in a small amount of the production medium and checked for the desired fermentation product and the normal fermen-