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Mixed Culture Fermentations

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PREFACE

The presentation of a symposium on mixed culture fermentations at Queen Elizabeth College, London (December 1980) coincided with a renewed interest in and awareness of many aspects of biotechnology. This volume consists of reviews based on papers delivered at the Symposium, which was organized by the Fermentation Group of the SGM.

The modern fermentation industry has evolved largely from the development of technology capable of maintaining large scale monocultures. This includes processes for vitamins, amino and other organic acids and antibiotics. Publications in 1976 describing the Shell process for the production of edible protein from methane-utilizing microbial communities were, therefore, regarded as revolutionary in concept. Unfortunately, it was not possible to obtain a further contribution on this process for the Symposium but many other applications of mixed culture fermentations to biotechnology are presented here.

The first two chapters attempt conceptual and kinetic analyses of the interactions which occur in mixed cultures. The remainder of the book is devoted to descriptions of waste treatment processes and examples of mixed culture usage in the manufacturing industries. The involvement of microbial communities in waste disposal is such a wide ranging topic that we have differentiated between the principles involved in aerobic and anaerobic processes.

Developments in the fermentations for yogurt and beer manufacture are described, followed by a speculative account of the involvement of the yeast-lactobacillus interactions in the production of a number of "traditional" fermented foods and beverages.

Finally an aspect of the programme, under development at Queen Elizabeth College, for the microbial assimilation of solar energy is described.

This book has been edited by myself and Dr.J.H. Slater (University of Warwick) who also wrote the first Chapter, which introduces the subject.

M.E. Bushell
December 1980
University of Surrey.

CONTENTS

Chapter 1

MIXED CULTURES AND MICROBIAL COMMUNITIES

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1. Introduction

For most microbiological studies and processes mixed cultures are considered to be the antithesis of good experimental techniques and practice. Microbiology students are rightly inculcated with the principles of pure culture techniques since much of our understanding of the properties and behaviour of microbes stems from work with axenic cultures. This is a necessary approach and tradition which, of course, remains as the cornerstone of experimental microbiology. It is due to the unique insight of Robert Koch who, one hundred years ago, published the first account of the method which reliably enabled him to isolate and maintain microbial cultures containing a single species. The method depended on microbial growth on the surface of a suitable, solid growth medium, the formation of microbial colonies starting from a single cell and, in principle and practice, remains unchanged to this day [Koch, 1881]. The paper was entitled "Methods for the study of pathogenic organisms" and fittingly started on page one of volume one of the "Reports of the Kaiser's Health Office". In the introduction to this seminal report Koch said that he did "not believe it is too much to say that the most significant point in all studies on infectious diseases is the use of pure cultures" and in writing this drew attention to the major problems that microbiologists then faced: namely, the inability to resolve microorganisms into species and correlate different species with different diseases and processes. Indeed at that time many bacteriologists thought that there was only one type of microbe and that different diseases were manifestations of different properties of the same organism.

The fact that the nineteenth century bacteriologists strove so hard to develop a reliable isolation and pure culture technique, highlights a truism which is greatly underestimated because of our insistence upon the use of

axenic cultures: that is, pure culture growth systems are highly unrepresentative of almost all the habitats which support the growth of microorganisms. Most natural habitats contain a wide diversity of microorganisms, even those habitats which, for our convenience and with our prejudicies, are defined as extreme. For example, the Dead Sea which has a salinity which is at least 10 times greater than that of the oceans, has been shown to support the growth of at least 10 bacterial species [Brock, 1969] and this number is several orders of magnitude lower than the species composition of most so-called normal habitats. The structural and functional diversity of microorganisms obtained from the same habitat is due, in part, to the heterogeneity of the habitat and reflects the fact that specific microbes have evolved to occupy successfully that characteristic niche. Nevertheless, microbiologists, and microbial ecologists in particular, have been aware for a long time that the "growth range" of different microorganisms overlap, despite the spatial and temporal properties of a particular habitat. This is a situation which can and does lead to a variety of basic types of interaction between different species [Slater and Bull, 1978]. It is our argument [Slater, 1978; Slater and Lovatt, 1981; Bull, 1980] that there may be important principles and properties of mixed microbial cultures which may have been overlooked and neglected in the past. This is clearly of considerable importance to microbial ecologists studying the capabilities of microbial communities in their natural habitats or in laboratory-based systems which simulate various features of the natural environment. But the properties of mixed culture growth may be of potential interest to the microbiological processing or biotechnological industries and these may not yet have been fully exploited. At the outset it has to be recognized that most high technology processing industries (e.g., antibiotic production, primary and secondary metabolite production and others) rely on pure culture systems, for which there may be no need for or advantage to mixed cultures. In others, notably the food and, to some extent, the beverage industries, mixed culture systems are necessary and traditionally used [see Driessen, White and Kidney, and Wood, this volume]. In most of these cases the mechanisms involved with mixed culture growth remain obscure.

In this chapter I shall review briefly some of the principles of interacting mixed cultures although the definitions of the various basic types of interactions are dealt with elsewhere [Bungay and Bungay, 1968; Meers, 1973; Slater and Bull, 1978; Kuenen and Harder, 1981; see Bazin this volume]. In particular this chapter seeks to outline some of the difficulties, limitations and consequences of mixed cultures and microbial communities.

2. Types of Microbial Community

There is a growing literature describing many categories of interacting assemblages of microorganisms, although one of the present difficulties is to know exactly how significant some of these associations may be. Furthermore there is considerable confusion over the terminology used to describe the interactions and communities.

In general terms there is a spectrum of microbial communities ranging from rather tenuous, "loose" associations to "tight" associations, a few of which appear to be obligatory. In one sense any environment which supports the growth of more than one population constitutes a community and, to a lesser or greater extent and at different times and under different conditions, the component populations inevitably interact. This may not occur at all times but may, for example, be restricted to periods when a particular nutrient is present at limiting concentrations and affects the rate of growth of more than one population. Although such circumstances may be particularly important from an ecological viewpoint, there is little of detailed significance in the species composition. Many of these loose associations depend on non-specific commensal relationships and, in many cases, simply describe the flow of carbon, energy and other growth requirements between different populations.

The communities at the other end of the range are, in the present context, more interesting, exhibiting properties and characteristics which may not be apparent with any other combination of microorganisms or may not be expressed by the component populations existing alone or both. For example, the complete mineralization of a compound may require the sequential metabolism of two or more organisms with the component populations alone being unable to transform the compound since, separately, they do not possess the complete genetic complement to code for the whole biodegradative pathway [Slater and Godwin, 1980]. These tight associations will be referred to as microbial communities, although elsewhere they are also known as consortia [Whittenbury, 1978], syntrophic associations [Pfennig, 1978] or synergistic associations as well as simply mixed cultures or interacting assemblages.

It is now possible to suggest a simple classification of the various microbial communities which have been isolated [Slater, 1978; Slater and Lovatt, 1981] (Table 1). This classification has been compiled on the basis of the approach various workers have taken to the study of microbial communities and one of the major difficulties at the moment is that in most cases the analysis of these communities is often incomplete. Furthermore, with a more detailed understanding of the

TABLE 1

Classes of Microbial Communities [after Slater and Lovatt, 1981]

1.	Structure due to the provision of specific nutrients between different members of the community.
2.	Structure due to the removal of metabolic products which may be inhibitory to the producing member of the community, including hydrogen transfer communities.
3.	Structure and stability due to interactions which may result in the modification of individual population growth parameters resulting in a more competitive or efficient community (compared with component populations).
4.	Structure due to the effect of a concerted, combined metabolic capability, not expressed by the individual populations acting alone.
5.	Structure due to a cometabolic stage.
6.	Structure due to the transfer of hydrogen ions.
7.	Structure is the result of the presence of more than one primary substrate utilizer – in many cases the nature of the interactions are unknown.

exact nature of interactions, particularly with categories 3 and 7 (Table 1), some microbial communities may be placed in other classes. For example, some stable communities have been examined with respect to some basic growth parameters including the maximum specific growth rate, saturation constant, inhibition constants and others [Osman *et al.*, 1976; Bull and Brown, 1979]. In the case of a three-membered orcinol community [Osman *et al.*, 1976] the nature of the interaction between the primary orcinol utilizer and its two associated non-orcinol utilizers was not determined. However, it is probable that the three-membered community's effectiveness, in growth kinetic terms, was due to the removal of inhibitory products generated as a result of the metabolism of the primary pseudomonad. Thus this community may simply be another class 2 type.

2.1. *Class 1 Microbial Communities*

The class 1 communities are widely distributed, readily isolated and most of those studied so far involve either commensal or mutualistic relationships dependant on the provision or requirement of growth factors or amino acids. This type of mixed culture is sometimes said to exhibit syntrophism. A number of examples of this category of community are shown in Table 2 and some of the reasons

why these communities seem to occur so readily are discussed in Section 3.2.

2.2. *Class 2 Microbial Communities*

Class 2 microbial communities are those where excreted materials, which may be toxic and growth inhibitory to the producing organism, are consumed by associated members of the community. A number of these communities have been well characterized [Wilkinson *et al.*, 1974; Cremieux *et al.*, 1977] when methane or methanol are used as the primary carbon and energy sources (Fig. 1).

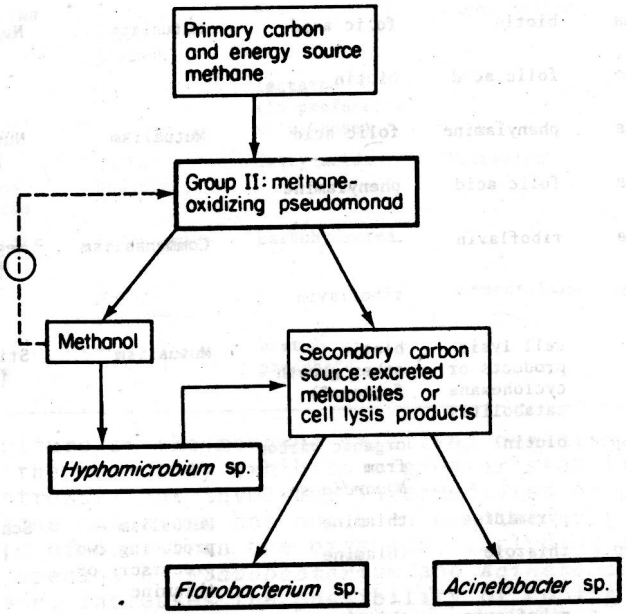

Fig. 1. A four-membered methane-utilizing microbial community illustrating the role of one population in removing an inhibitory metabolite produced by another organism (Wilkinson *et al.* [1974] after Slater [1978]).

The effectiveness of these communities was well illustrated by the four-membered community containing the *Hyphomicrobium* species, present to metabolize excreted methanol. If the mixed culture was stressed by the addition of extra methanol, methane oxidation by the primary pseudomonad ceased and there was an increase in the proportion of *Hyphomicrobium* sp. cells in the mixture: under the conditions examined the increase was from 4% to 25%. This ensured the rapid reduction of the methanol concentration, a resumption of methane oxidation and a gradual restoration of the original community composition. Clearly this community was more stable than a

TABLE 2

Class 1 Microbial Communities with Specific Nutrient Requirements

Organism	Compound		Type of interaction	Reference
	product	requirement		
Bacterium 3C1	—	Vitamin B_{12}	Commensalism	Jensen [1957]
Streptomyces spp.	Vitamin B_{12}	—		
Streptococcus faecalis	biotin	folic acid	Mutualism	Nurmikko [1954]
Lactobacillus plantarum	folic acid	biotin		
Streptococcus faecalis	phenylamine	folic acid	Mutualism	Nurmikko [1956]
Lactobacillus arabinosus	folic acid	phenylamine		
Saccharomyces cerevisiae	riboflavin	—	Commensalism	Megee *et al.* [1972]
Lactobacillus casei	—	riboflavin		
Nocardia sp.	cell lysis products or cyclohexane catabolites	biotin (+ other growth factors?)	Mutualism	Stirling *et al.* [1976]
Pseudomonas sp.	biotin	organic carbon from *Nocardia* sp.		
Mucor	pyrimidine	thiamine	Mutualism — producing two precursors of thiamine	Schopfer [1943]
Rhodotorula sp.	thiazole	thiamine		
Marine bacterium sp.1	riboflavin	thiamine	Mutualism with precursors	Burkholder [1963]
Marine bacterium sp.2	pantothenate	thiamine		
Bacillus polymyxa	biotin	nicotinic acid	Mutualism	Yeoh *et al.* [1968]
Proteus vulgaris	nicotinic acid	biotin		
Auxotrophic algae	—	vitamins	Commensalism	Carlucci & Bowes [1970]
Heterotrophic bacteria	vitamins	—		
Saccharomyces cerevisiae	nicotinic acid	—	Commensalism	Shindela *et al.* [1965]
Proteus vulgaris	—	nicotinic acid		

TABLE 2 (cont'd)

Organism	Compound		Type of interaction	Reference
	product	requirement		
Acetobacter suboxydans	fructose (from manitos)	–	Commensalism	Chao & Reilly [1972]
Saccharomyces carlbergansis	–	fructose		
Lactobacillus plantarum	lactate (from glucose)	–	Commensalism	Lee *et al.* [1976]
Propioni-bacterium shermanii	–	lactate (in preference to glucose)		
Rhodo-pseudomonas capsulatus	carbo-hydrates	fatty acids	Mutualism	Okuda & Kobayoshi [1963]
heterotrophic bacteria	fatty acids	carbohydrates		
Diphtheroid	acetyl-phosphate	–	Commensalism	Nevin *et al.* [1960]
Borrelia vincenti	–	acetyl-phosphate		

pure culture of the methane-oxidizing organism. Whether or not the community exhibits a greater stability to other stresses not involving intermediates or products of methane oxidation has not been examined but the presence of more than one organism, including the peripheral species of *Flavobacterium* and *Acinetobacter* in this case, increases the possibility of coping with various stresses.

More recently Jones and Hood [1980] have demonstrated the importance these relationships may have with respect to particular metabolic activities of the primary organisms. As these authors point out "it is well recognized but not especially well documented" (and it might be added, usually ignored) that it is difficult to isolate nitrifying bacteria without accompanying heterotrophic populations and that the presence of the heterotrophs ensures better growth and increased rates of nitrification. This interaction may well be common and important for all chemolithotrophs, especially those which have an obligate mode of nutrition [Whittenbury and Kelly, 1977]. Jones and Hood [1980] described a three-membered community isolated from an estuarine environment containing an ammonium-oxidizing *Nitrosomonas* sp, associated with two heterotrophs, *Nocardia atlantica* and a species

of *Pseudomonas*. The heterotrophic organisms were shown to be incapable of heterotrophic nitrification. After growth as a mixed culture the level of nitrite produced by the *Nitrosomonas* sp. was 150% greater than the organism growing in the absence of the heterotrophs. Furthermore the growth of the heterotrophs was stimulated by a factor of 10 by the presence of the chemolithotroph. It is possible that the stimulation of nitrification was due to the production of required metabolites for the *Nitrosomonas* sp. but it is more likely, however, that the stimulation was as a result of the removal of organic compounds, toxic to the *Nitrosomonas* sp. and a known problem for the growth of fastidious chemolithotrophs [Pan and Umbreit, 1972; Whittenbury and Kelly, 1977].

2.3. *Class 3 Microbial Communities*

Class 3 microbial communities may, as has previously been suggested (page 4), be the consequences of a number of nutritional interactions affecting the overall growth kinetics. However, it is still possible that other types of interactions, especially those involving physico-chemical properties, may improve the growth kinetic parameters of the community compared with component populations. For example, there is some evidence that floc formations influence kinetics.

2.4. *Class 4 Microbial Communities*

Class 4 microbial communities are frequently encountered and are characterized by a structure which is summarized in Table 3. Under the conditions in which these associations are isolated, the interactions normally establish a mutualistic relationship: that is, the survival and growth of the community depends on the concerted activity of two or more populations. However, under other growth conditions, the metabolic relationships may not be required or expressed and, for this reason, these mixtures are sometimes said to exhibit synergism or protocooperation, implying that the mutualism is not obligatory.

So far most combined metabolism communities studied have exhibited mutualism in terms of biodegradative functions (Table 4) although this is not always the case. For example, Gale [1940] demonstrated that the synthesis of putrescine from arginine was due to the concerted metabolism of *Streptococcus faecalis* and *Escherichia Coli* (Fig. 2).

Pickaver [1976] also demonstrated that combined metabolism by several organisms may result in the synthesis of a product which can accumulate. A perfusion enrichment culture, with nitriloacetate (NTA) as the

TABLE 3

The Principle of Class 4 Microbial Communities
[after Slater and Godwin, 1981]

Pathway

$$A \xrightarrow{a} B \xrightarrow{b} C \xrightarrow{c} D \xrightarrow{d} E$$

Organism	Capability
X	Requires E (or a derivative of E) for growth. No growth on A, B, C or D. Produces enzymes a and b. Intermediate C accumulates from either A or B. C and D not metabolized.
Y	Requires E (or a derivative of E) for growth. No growth on or transformation of A or B. Produces enzymes c and d. Growth on C, D and E.
X + Y	Requirement for E may be satisfied by combined metabolism of A. Growth on A, B, C, D or E. Complete pathway present since the community contains enzymes a, b, c and d.
Z	Growth on A, B, C, D or E. Complete pathway present since organism contains enzymes a, b, c and d as a result of the transfer of genes for enzymes a and b from organism.
Z^1	X to Y Growth on A, B, C, D or E. Complete pathway present since organism contains enzymes a, b, c and d as a result of transfer of genes for enzymes c and d from organism Y to X.

carbon source and sodium nitrate as the nitrogen source, produced a five-membered community consisting of three pseudomonads (*Pseudomonas* sp. strains A, B or C), a *Bacillus* sp. and a yeast. After a period of growth some of the NTA was converted to *N*-nitrosoimino-diacetate (NIDA), probably as a result of an interaction between intermediates of NTA metabolism, namely iminodiacetate (IDA), and nitrate metabolism, namely nitrite. Analysis of the community showed that none of the isolates alone produced NIDA when incubated with NTA and nitrate. But two two-membered mixed cultures (*Pseudomonas* strain A with *Pseudomonas* strain B or *Pseudomonas* strain A with *Bacillus* sp.) could produce NIDA from NTA and nitrate.