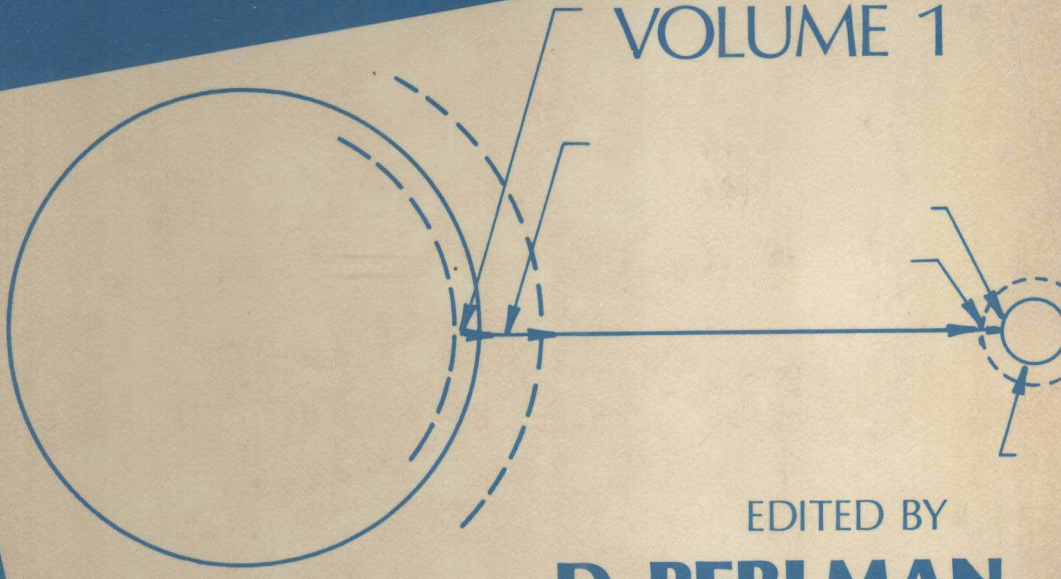
Annual Reports on Fermentation Processes

VOLUME 1



EDITED BY

D. PERLMAN

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D. PERLMAN

School of Pharmacy
University of Wisconsin
Madison, Wisconsin

ASSOCIATE EDITOR

GEORGE T. TSAO

School of Chemical Engineering
Purdue University
West Lafayette, Indiana

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List of Contributors

Numbers in parentheses indicate the pages on which authors' contributions begin.

BERNARD J. ABBOTT (205), Microbiological and Fermentation Products Research, Lilly Research Laboratories, Indianapolis, Indiana

K. AUNSTRUP (181), Microbiological Research and Development, Novo Industri, DK-2880 Bagsvaerd, Denmark

F. R. BERNATH (235), Department of Chemical and Biochemical Engineering, Rutgers University, New Brunswick, New Jersey

L. T. CHANG (1), Industrial Division, Bristol-Myers Company, Syracuse, New York

M. CHARLES (365), Department of Chemical Engineering, Lehigh University, Bethlehem, Pennsylvania

P. S. S. DAWSON (73), Prairie Regional Laboratory, National Research Council, Saskatoon, Saskatchewan, Canada

D. D. DOBRY (95), The Upjohn Company, Kalamazoo, Michigan

R. P. ELANDER (1), Industrial Division, Bristol-Myers Company, Syracuse, New York

M. GORMAN (327), Lilly Research Laboratories, Indianapolis, Indiana

F. HUBER (327), Lilly Research Laboratories, Indianapolis, Indiana

J. L. JOST (95), The Upjohn Company, Kalamazoo, Michigan

MASAKAZU KIKUCHI (41), School of Pharmacy, University of Wisconsin, Madison, Wisconsin (Present Address: Central Research Laboratories, Takeda Chemical Industries, Ltd., Osaka, Japan)

K. KIESLICH (267), Department of Mikrobiologische Chemie, Schering AG, D-1000 Berlin, West Germany

ALLEN I. LASKIN (151), Corporate Research Laboratories, Exxon Research and Engineering Company, Linden, New Jersey

YOUNG HIE LEE (115), School of Chemical Engineering, Purdue University, West Lafayette, Indiana

JAROSLAV MAJER (347), Department of Biochemistry, Medical and Dental Schools, Northwestern University, Chicago, Illinois

TAKASHI NARA (299), Tokyo Research Laboratory, Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan

L. K. NYIRI (365), Department of Chemical Engineering, Lehigh University, Bethlehem, Pennsylvania

D. PERLMAN (41), School of Pharmacy, University of Wisconsin, Madison, Wisconsin

COLIN RATLEDGE (49), Department of Biochemistry, The University of Hull, Hull, England
O. K. SEBEK (267), The Upjohn Company, Kalamazoo, Michigan
GEORGE T. TSAO (115), School of Chemical Engineering, Purdue University, West Lafayette, Indiana
R. W. VAUGHN (1), Industrial Division, Bristol-Myers Company, Syracuse, New York
K. VENKATASUBRAMANIAN (235), Department of Chemical and Biochemical Engineering, Rutgers University, New Brunswick, New Jersey
W. R. VIETH (235), Department of Chemical and Biochemical Engineering, Rutgers University, New Brunswick, New Jersey

Preface

ANNUAL REPORTS ON FERMENTATION PROCESSES is designed to furnish readers with a critical account of significant developments published during the past two to three years concerning fermentation processes. Only published material is included, and the main value of the volumes in this series is to assist the reader to keep abreast of developments in areas of fermentation research and developments where he has only peripheral or limited interest. The contributors of chapters to this volume were asked to answer the question "What are the *major* developments in the field published recently?" and have done so very admirably.

Many persons are involved in decisions in launching a new series and we are indebted to them for assisting in this process. The officers past and present of the Division of Microbial and Biochemical Technology of the American Chemical Society have been very helpful in getting this project started, as has the publisher, Academic Press. We hope that the first volume will meet readers' needs and we will appreciate suggestions on modifications for future volumes.

May 20, 1977

D. Perlman

School of Pharmacy
University of Wisconsin
Madison, Wisconsin

Introduction

The Division of Microbial and Biochemical Technology is extremely pleased to sponsor the publication of "Annual Reports of Fermentation Processes" under the capable editorship of Professor David Perlman. For the first time, a broad cross section of subjects of importance and interest to our members will be reviewed by experts in the field on a regular basis. With this first volume printed in 1977, the Divisional Executive Committee wishes all the success in this new and hopefully lasting activity. We would also thank Academic Press, Inc., for its support and cooperation.

George T. Tsao, Chairman

Division of Microbial and
Biochemical Technology
American Chemical Society

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CHAPTER 1

GENETICS OF INDUSTRIAL MICROORGANISMS

R. P. ELANDER, L. T. CHANG, AND R. W. VAUGHAN

Bristol-Myers Company
Industrial Division
Syracuse, New York

Although basic studies on the genetics and molecular biology of microorganisms have dramatically expanded our understanding of microbial regulation of primary metabolism in a few well studied organisms, relatively little attention has been focused on the molecular and genetic aspects of industrially important microorganisms. During the past two decades, basic genetics and the applied genetics of industrial organisms have gone in somewhat diverging directions. Although mutation and selection programs were highly successful, basic new genetic methodology was not easily adopted in industry for a variety of reasons including a lack of appreciation for industrial problems by academic geneticists, a lack of basic understanding of the genetic and recombination mechanisms in industrial strains, and a lack of competent geneticists in the fermentation industry. In order to help bridge this gap, a series of important genetic conferences--Genetics of Industrial Microorganisms (GIM)--were held in Prague (1970), Sheffield (1974), Orlando (1976) and Madison (1978). These conferences were organized to review recent approaches to tailor microorganisms for practical uses, to expand and include industrially important microorganisms for which genetic studies have been conducted, and to review recent applications of molecular biology and genetics in strain improvement. The proceedings of these highly successful conferences are now published and offer a comprehensive review of topics related to microbial genetics (1-3) Other important genetic topics are covered in other publications and reviews (4,5).

I. MUTATION, SELECTION AND OPTIMIZATION OF MUTAGENESIS

Mutation in microorganisms is thought to arise through two major classes of mutagenic mechanisms: errors directly induced through base mispairing or errors introduced by the various repair mechanisms. Direct induction of mutations can result from the alkylation of guanine and thymine by agents such as ethyl methanesulphonate (6-9). However, the majority of mutations are determined largely by excision, postreplication, and error-prone repair systems acting on single-strand gaps formed directly or indirectly in DNA.

The excision repair process excises a lesion in one strand of the DNA duplex and the resulting gap is filled by a DNA polymerase enzyme using the undamaged strand as a template. Strains that are difficient in excision repair are more apt to give rise to mutations than excision repair-proficient strains. A more practical, but less efficient procedure for the industrial microbiologist is to use compounds such as caffeine (11-12) and acriflavine (13,14) which inhibit the excision repair process as part of the mutagen regimen. Induction of mutations by the postreplication repair process involves DNA replication and recombination. Gaps ($\sim 10^3$ bases) are formed in daughter strands opposite induced lesions in the parental strands (9,15,16). The majority of gaps are filled by a recombination process (17) between the two daughter chromosomes (18). The process is complex since photo-induced dimers are not inherited lineally but are randomly distributed among the progeny strands (19). An error-prone repair system, which probably does not involve recombination, may operate as an alternative pathway simultaneously with the excision and postreplication repair processes. The postulated mechanism is incorrect insertion of bases into gaps in the progeny strand DNA but the process is not fully understood (9,20-24). Bridges (25) reported the enzyme DNA polymerase III is necessary for error-prone repair but is not required for postreplication or excision repair. Mutations induced through the above mechanisms are generally base-pair substitutions. Frameshift mutations result from the addition or removal of base pairs in multiples not equal to three which causes the translation process to lose the proper frame of reference (9,26).

A converse of mutation induction, the detection of mutagenic compounds, has recently emerged as an area of importance to the industrial microbiologist. Government regulations are requiring increased testing of industrially important compounds for mutagenic and carcinogenic activity. Ames et al. (27-30) have developed sensitive bacterial testor strains which detect both base substitution and frameshift mutagens. A few of the common mutagens are cited here and the reader is referred to Fishbein et al. (31) for a more comprehensive treatment. Ultraviolet light, ionizing radiations (9,24,32-34) and heat (9) are probably the safest agents to use but alkylating agents (33-38), 4-nitroquinoline-1-oxide (39,40) and sporalens (41,42) in the presence of 360 nm light are sometimes preferred.

Optimization of mutation induction involves the interaction of pH (35,36) concentration (37) period of treatment, phase of cellular growth, and the interactions are usually organism-dependent. Incorporation or elimination of DNA precursor bases can also affect mutation rates (43,44). Addition of compounds which inhibit the excision repair mechanism increase the effectiveness of ultraviolet light (11,12,14). Analysis of the interactions requires histograms or biometric considerations (45) when random isolation of mutants are used. A simpler and more precise analysis can be obtained with dose-effect plots (48) when specific selection methods are employed (e.g. mutation to resistance or reversion of auxotrophs to prototrophs). However, the optimum procedure determined for resistance or reversion mutants does not always coincide with the optimum procedure required for increased product formation of compounds of interest to the industrial microbiologist.

Selective isolation of biochemical mutants from a mutagenized population is perhaps the most difficult task in a mutant isolation process. Methods are now available which offer distinct advantages over the total isolation process. Replica plating (46) works well with bacteria and fungi which produce spores or fragmented hyphae. Filamentous organisms can be successfully manipulated by the addition of compounds to agar which induce microconidia, spores, fragmented cells or restrict colony size. The addition of 0.08% sodium deoxycholate to agar induces *Aspergillus nidulans* to produce restricted colonies (34) while sorbose restricts the colonial growth of *Neurospora* (47). Dense velveteen pads of closely set steel needles or filter paper discs can be used as successful transfer vehicles. Layering techniques (48) are useful with yeasts. An appropriately diluted population of mutagenized cells is plated on minimal agar. Prototroph cells give rise to small colonies after a period of incubation and a layer of complete medium is then poured over the original medium. Auxotrophs begin to grow and can be differentiated by their size difference or the prototrophs can be marked before addition of the overlay medium.

Resistance mutants can be isolated by plating large concentrations of mutagenized populations on solid media containing a toxic substance. Isolation of prototrophic reversions from auxotrophs requires plating only on a minimal medium. Enrichment techniques based on selective elimination of growing cells before plating on isolation media increase the probability of isolating biochemical mutants. The procedure can be made specific if compounds are added to the media which allow all but the desired mutants to grow. Filtration procedures, first reported by Fries (49) are primarily applied to filamentous organisms (50-52). Penicillin enrichment, originally developed for bacteria (53) is being widely used with modifications (54-58). The principle of penicillin enrichment can be extended to the isolation of auxotrophs for many organisms by the use of compounds which have selective toxicity. Sodium pentachlorophenate is harmless to spores of *Penicillium chrysogenum, Streptomyces aureofaciens, S. olivaceus* and *Bacillus subtilis* but is lethal to their germinated spores.

(59,60) Nystatin is effective with *P. chrysogenum* (61,62) yeasts (63) and *Cephalosporium acremonium;* 2-deoxyglucose with *Schizosaccharomyces pombe* (64) and netropsin with *Saccharomyces cerevisiae* (65). Certain mutations confer reduced viability to organisms when starved in a minimal medium and induction of a second mutation restores survival. This technique has been used to advantage, for example, with inositol (66) and thymine (67,68) mutants for the isolation of additional auxotrophic markers. There are instances where specific compounds select specific mutations. Trimethoprim selects for thymine-requiring mutants (69,70) in bacteria. The specific action of 4-nitropyridine-1-oxide effectively selects proline-requiring mutants in *E. coli* (71).

A chemostat offers great promise if selective pressure can be applied which gives even a minimal growth advantage to the desired mutant. Monitoring the steady-state population metabolite concentrations in the effluent permits continuous control of the environment and development of a mutant population. Mutants of *E. coli* constitutive for β-galactosidase (72) *P. putida* constitutive for mandelate enzymes (73) *K. aerogenes* with altered xylitol to ribitol activity ratio of ribitol dehydrogenase (74) and *S. cerevisiae* with an acid phosphatase with an altered pH optimum (75) have been successfully isolated from a chemostat culture. The method has application to more than selection of cultures which overproduce enzymes or have altered enzyme specificities.

Mutants resistant to metabolic analogues can be isolated which are derepressed, resistant to feedback inhibition, or altered in regulation of branched or secondary metabolic pathways. More detail can be found elsewhere in this chapter or in review articles (76-78).

II. MUTATION AND IMPROVED PRODUCT YIELD IN ANTIBIOTIC-PRODUCING MICROORGANISMS

Mutation and selection to increased product formation is probably the most important factor in improving the yield of an antibiotic (79). Mutation programs continue to be vital to the fermentation industry in that mutation to higher productivity in periods of increasing labor and raw material costs is the most important factor in maintaining the industry in an economically healthy state. Intensive strain improvement and concurrent genetic programs are being expanded in both industrial and applied laboratories throughout the world.

A. Mutagenic Treatment, Morphological and Biochemical Variants, and Antibiotic Productivity

The most effective mutagen for improving tetracycline productivity in strains of *Streptomyces aureofaciens* is UV radiation. Of the mutagens tested, N-methyl-N'-nitro-N-nitrosoguanidine (NG)

was also effective, whereas X-ray and nitrogen mustards were relatively ineffective with γ-radiation inducing the greatest number of nonactive variants (80). Strains of *S. aureofaciens* and *S. rimosus* show considerable morphological variation following mutagenic treatment with most morphological and biochemical variants possessing a decreased potential to synthesize tetracyclines. Morphological types present in the penicillin fungus, *Penicillium chrysogenum*, collectively termed the "population pattern phenomenon" proved to be useful in the historical development of the Wisconsin family of improved penicillin-producing variants (81). This family of strains provided the basis for subsequent yield improvement programs with penicillin fungi (4).

Auxotrophic mutation generally leads to lower productivity in antibiotic-producing microorganisms, especially when mutants are selected for deficiencies in factors which are also precursor metabolites for antibiotic synthesis. Auxotrophic mutants of *P. chrysogenum* exhibited markedly decreased vegetative development and antibiotic yields varied from 60 to 90% of the wild-type culture (82). The results of a detailed study with strains of *Cephalosporium acremonium* revealed a decrease in relative potency with increasing mutagenesis. An improved variant of *C. acremonium*, CW-19, produced nearly a 15-fold improvement in cephalosporin C and a significantly improved cephalosporin C/penicillin N ratio compared to either the Brotzu strain or the M-8650 variant (83). Higher ploidy strains of cephalosporin fungi and strains resistant to polyene antibiotics have also been reported to synthesize increased levels of cephalosporin C. Strains blocked in the sulfate reduction pathway were reported to assimilate more exogenous methionine and to synthesize fourfold more cephalosporin C than the sulfide-proficient parent (84). Nüesch et al. (85) proposed that mutants blocked in the sulfate-reduction pathway were incapable of synthesizing cysteine, a repressor and inhibitor of methionine permease. Queener et al. (86) reported that the specific activity of glutamate dehydrogenase was derepressed in high-yielding strains which may have removed a nitrogen limitation for cephalosporin C synthesis. Recent reviews focusing on industrial strains improvement programs with antimycins (87) carminomycin (88) cephalosporins (89) and other antibiotics (90) show continuing progress in this important aspect of fermentation development.

B. Reverse Mutation Variants

Dulaney and Dulaney (91) obtained revertants from nonproducing strains of *Streptomyces viridifaciens* which produced more than a six-fold increase in chlortetracycline compared to their grandparent. Overproduction of precursor molecules is another means to achieve increased antibiotic formation. The overproduction can be achieved by relieving feedback regulation of its biosynthetic pathway. Modification of the structure of a feedback-sensitive enzyme through auxotrophic mutation followed by replacement of a

second reversion (suppressor mutations) is a common method for relieving this regulation. Reverse mutation of methionine auxotrophs resulted in a significant increase in chlortetracyline yield compared to the original prototrophic strain (91).

C. Mutation to Antibiotic Resistance by Producer Strains

Antibiotics may be toxic to growing cultures of producer organisms. Antibiotics which inhibit the growth phase of producer strains include tetracycline, novobiocin, actinomycin, streptomycin and nystatin. Differences in biosynthetic capacity may be affected by resistance to their own antibiotic. Therefore, the possibility to increase resistance by adaptation to increasing concentrations of antibiotic is a useful procedure toward improving the productivity of strains. Katagiri (92) obtained a fourfold increase in productivity in *S. aureofaciens* by repeated transfers of a strain to increasing concentrations of chlortetracycline. The results can be explained by the lethal effect of the antibiotic which selectively deprives the natural population of low active sensitive clones. This method is particularly effective during the initial phase of strain improvement or in cases when the population of a highly productive strain must be repurified (79).

D. Mutation to Resistance to Precursors or Analogues of Precursors

The important precursor molecule of the penicillin V fermentation is phenoxyacetic acid, a mildly toxic compound which stimulates strains of *P. chrysogenum* to generate penicillin V in copious amounts at the expense of other naturally occurring penicillin moieties. There appears to be a rather direct relationship between degree of penicillin V accumulation and the capability of the strain to make growth in the presence of increasing concentrations of precursor. Modern production strains are capable of detoxifying the precursor by incorporating the precursor into the side chain of the penicillin.

Mutants selected for resistance to analogues of primary metabolites often overproduce end-product since the strains are no longer subject to feedback repression. This concept has been applied to the pyrrolnitrin, an antifungal antibiotic synthesized by a number of pseudomonads (93). D-tryptophan is a precursor of this antibiotic and is stimulatory for antibiotic formation. The high cost of the amino acid made it impractical for use. Analogue-resistant mutants to fluoro- and methyl-tryptophan were no longer subject to feedback inhibition and resistant mutants were selected which produced nearly threefold more pyrrolnitrin than the parent strain in the absence of added tryptophan (94,95).

III. MUTATION AND SELECTION SYSTEMS IN NONANTIBIOTIC-PRODUCING INDUSTRIAL MICROORGANISMS

Induced mutations as a means to improve product yields have been employed since the beginning of modern fermentation industries. In addition to random testing of survivor colonies for improved product yield in broth cultures, the most commonly used criteria for selection is morphological mutation. There are abundant examples of success with the use of morphological mutants for improved yield (77,96). However, as our understanding of biosynthetic pathways and enzyme regulation has increased, more sophisticated and rational selection methods were developed to suit the needs of individual fermentation products. The following discussion deals with the more important mutation and selection methods used for strain improvement in nonantibiotic-producing microorganisms.

A. Analogue-Resistant Mutants

The isolation of analogue-resistant mutants involves plating of mutagenically-treated cell populations (>10^5/plate) on a medium containing toxic analogues of precursors or end products. This method has been used successfully for improved amino acid production in strains of *Corynebacterium glutamicum* (97,98) and *Saccharomycopsis lipolytica* (99): increased production of histidine, tryptophan, lysine, phenylalanine and methionine as a result of mutation to increased resistance to the respective amino acid analogues. In many cases, the sequential increase in level of resistance correlated with increased level of productivity (97). The rationale for such method was that the analogue-resistant mutants were rendered insensitive to end product inhibition (77).

B. Auxotrophs

Auxotrophs have been widely used for the production of amino acids involved in branched pathways by *C. glutamicum* (77,97,98). The lysine-producing strain is a homoserine (or threonine and methionine) auxotroph; this was made possible by blocking that part of the branched pathway leading to methionine, threonine and isoleucine. The strain used for the production of glutamic acid was a natural biotin auxotroph. On the other hand, amino acid auxotrophs are generally poor producers of industrial enzymes and citric acid (100). However, there was a report that the production of citric acid was enhanced in a glutamate auxotroph of *Saccharomyces cerevisiae* (101).

C. Direct Selection on Agar Plates

A method similar to the bioassay plate for detecting antibiotic is widely used for detection of many enzymes and citric acid by colonies on agar plates (102,103). Many hydrolytic enzymes, such as amylase, protease and lipases change the turbidity of the agar medium if the enzyme substrates such as starch, casein or lipids are incorporated into agar. Citric acid production by colonies can also be visualized on the agar plates when pH indicators or $CaCO_3$ are incorporated into the agar (104).

D. Miscellaneous Indirect Methods

Monofluoroacetate (MFA) is known to inhibit aconitate hydratase (which converts citrate to isocitrate) in *Candida lipolytica*. A mutant which becomes sensitive to MFA produced less aconitate hydratase than wild type strains. Such mutants, when used to ferment n-paraffins, produced citric acid and isocitric acid at a ratio of 97:3, instead of the usual ratio of 60:40 (105). A similar approach has been used to isolate mutants with increased ability to take up methionine for the production of cephalosporin C in *C. acremonium* (106). Such mutants, obtained by isolating seleno-methionine-sensitive mutants, were blocked in the assimilation of inorganic sulfur. A mutant strain of *Candida tropicalis* (as a source of single-cell protein) in which protein methionine content increased by 41% was obtained by selecting the smaller colonies on a sulfur deficient medium (107). The production of glucose isomerase (GI) by *Streptomyces spp*. (for conversion of glucose to fructose) was repressed by glucose and required the presence of D-xylose in the media as inducer. Mutants resistant to an analogue of xylose, i.e. D-lyxose, were found to produce (GI) constitutively (without xylose), but were still repressible by glucose. Additional selection for resistance to a glucose analogue, 3-0-methyl glucose, resulted in isolation of a mutant which produced GI even in the presence of glucose (insensitive to catabolite repression). (108). Finally, manipulation of parasexual cycle in fungi can be used to select for high yield strains. Heterozygous diploids and their haploid segregants of *Aspergillus spp*. have been reported to be superior to their haploid parents in the production of citric acid, fungal amylase, fungal protease and kojic acid (109,110).

IV. GENETIC MAPPING AND RECOMBINATION IN YEASTS

Successful breeding programs for strain improvement depend upon knowledge of life cycles and adequate genetic maps. The genetics of *Saccharomyces cerevisiae* (111,112), used in the brewing and baking industries, is the most definitive map available in yeasts. Extensive efforts with laboratory strains in defining ploidy, sporulation characteristics, ascospore viability, and