AN ABSTRACT OF THE THESIS OF

RONALD WAYNE BERG (Name) for the DOCTOR OF PHILOSOPHY (Degree)

in Microbiology (Major) presented on December 3, 1973 (Date)

Title: YEAST EXTRACTIVES IN THE NUTRITION OF SAN FRANCISCO SOURDOUGH BACTERIA (LACTOBACILLUS SANFRANCISCO)

Redacted for privacy

Abstract approved: Dr. A. W. Anderson

A laboratory-prepared hot-water extract of yeast is required for vigorous growth of San Francisco sourdough bacteria (Lactobacillus sanfrancisco) in pure culture. This requirement was examined with the goal of determining what is unique about fresh yeast extractives (commercial extractives exhibit much lower growth promoting activity), and which components of fresh yeast extractives are required or stimulatory in the nutrition of these bacteria.

Autolysis, the process by which most commercial extractives are prepared, was shown to be less efficient in releasing growth promoting materials for sourdough bacteria than the hot-water extraction procedure used for the preparation of fresh extractives.

Essential nutrients supplied by fresh yeast extractives were found to include manganous ion, which the organisms require at a concentration of about 0.2 μg/ml for maximal growth, several

vitamins and nucleic acid bases, and a short peptide which accounts for the unique growth promoting activity of this material. The four strains of sourdough organisms examined were found to require thiamine, nicotinic acid, and calcium pantothenate for growth in the test medium employed, while one strain requires, in addition, folic acid. One or more of the nucleic acid bases adenine, guanine, or uracil were demonstrated to be required or stimulatory for all strains. The peptide to which the unique growth promoting activity of fresh yeast extractives may be attributed was isolated and found to contain aspartic acid, cysteine, glutamic acid, glycine, and lysine, with a chain length of about nine residues and a molecular weight of approximately 1,065.

Yeast Extractives in the Nutrition of San Francisco Sourdough Bacteria (*Lactobacillus sanfrancisco*)

by

Ronald Wayne Berg

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

June 1974

APPROVED:

Redacted for privacy

Professor of Microbiology

Redacted for privacy

Head of Department of Microbiology

Redacted for privacy

Dean of Graduate School

Date thesis is presented December 3, 1973

Typed by Susie Kozlik for Ronald Wayne Berg

ACKNOWLEDGEMENTS

To my parents who supported and encouraged my educational goals, and to Dr. James M. Craig who gave direction and guidance which led to graduate study, I owe a large debt of graditude. Appreciation is extended to Dr. A. W. Anderson, a major professor who allowed me to "do my own thing" and make my own mistakes which are essential to the exercise and development of creativity. Thanks are due Dr. R. R. Becker and R. L. Howard for assistance with the amino acid analyses, and Dr. W. E. Sandine for reviewing the manuscript.

It is to my wife, Ann, for her boundless patience, understanding and love, which have made the meager life of a graduate student bearable, even fun, that I dedicate this thesis.

This work was supported by a grant from the USDA (No. 12-14-100-10313(74)) and by NDEA Title IV Fellowship No. 70-03024.1.

Any piece of knowledge I acquire today has a value at this moment exactly proportioned to my skill to deal with it. Tomorrow, when I know more, I recall that piece of knowledge and use it better.

-Mark Van Doren

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

YEAST EXTRACTIVES IN THE NUTRITION OF SAN FRANCISCO SOURDOUGH BACTERIA (LACTOBACILLUS SANFRANCISCO)

INTRODUCTION

San Francisco sourdough French bread has become a popular product of a unique local industry. A certain mystique has surrounded the process, the success of which has been variously attributed to such things as foghorns bleating forlornly through gathering mists and "little cable cars climbing halfway to the stars." Microbiologists reduced the magical aspects somewhat by discovering that the process owes its success to a yeast and a rather unique bacterium. The bacterium, however, has some magical qualities of its own, the study of which is the object of this thesis.

A great deal of difficulty was encountered in isolating bacteria from sourdough fermentations, a result of their fastidious nature and unknown nutritional requirements. For successful isolation, a laboratory-prepared, hot-water extract of yeast was required and could not be replaced by the commercially available extractives tested. This requirement was not only inconvenient and possibly expensive for large scale production of sourdough bacteria, but very puzzling as well. The research which lead to this thesis was directed toward answering the questions: (a) What is unique about fresh yeast extractives, and (b), Which component(s) of fresh yeast extractives are

required or stimulatory in the nutrition of sourdough bacteria. The goal of such studies is the development of background nutritional information for application to the technology of concentrated starter culture production. Such concentrates would not only simplify sourdough bread production, but allow export of the process to other areas as well. While a more conveniently prepared and less expensive alternative to fresh yeast extractives may be found for industrial-scale production of active sourdough bacteria, the necessity for nutritional information on a defined, molecular level is certainly not diminished for complete understanding and control of the process.

LITERATURE REVIEW

Microbiology of San Francisco Sourdough

San Francisco sourdough French bread has been produced for over 100 years in the San Francisco Bay area, where it has become a well-established and popular commodity not only on the local market, but also for export to other parts of the United States and foreign countries. Until recently, very little was known about the mechanical and microbiological aspects of the process.

The process relies on a starter, or mother sponge, as the source of leavening and souring power as well as a means for perpetuating the process. This starter consists of 100 parts of previous sponge, 100 parts of flour, and about 50 parts of water. After make-up, it is held at 27 C for seven to eight hours, during which time the pH drops from about 4.5 to 3.8 (Kline, Sugihara, and McCready, 1970). In commercial practice, this process is repeated every eight hours, seven days a week, and has presumably been accomplished in this manner since the industry began. The high proportion of previous starter sponge used in preparing a new one serves two purposes. First, it provides a massive inoculum of the microorganisms involved, and second, it provides a very acid environment which potentiates the self-protective nature of the system.

The bread dough is made up with the fully developed starter sponge (approximately nine to 15 percent), flour, water, and salt. During a proof period of six to eight hours at 30 to 32 C, the pH of the dough drops from about 5.3 to about 3.9, approximately the same pH reached by the starter sponge itself (Kline, Sugihara, and McCready, 1970). The final product is then produced by baking in steam injection ovens.

Initial attempts to isolate bacteria from starter sponges in appreciable numbers, using several dozen culture media designed for the isolation of a wide variety of bacteria, were unsuccessful (Sugihara, Kline, and McCready, 1970). However, these investigators were able to isolate a rather unusual species of yeast identified in part by its inability, unlike bakers' yeast, to ferment or grow on maltose. These microorganisms were demonstrated to be responsible for the leavening activity, but not the souring action of the sourdough starter, and were subsequently identified as asporogenous strains of Saccharomyces exiguus (Torulopsis holmii) (Sugihara, Kline, and Miller, 1971).

Microscopically, bacteria could be observed in large numbers in slurries made from doughs, and since the origin of the souring activity was still unresolved, renewed attempts were made to isolate them. The development of a medium for successful isolation of sourdough bacteria was based upon simulating some of the conditions

of the natural environment in the sourdough. A key discovery was that these bacteria would grow on or ferment only maltose and not on any of the usual sugars such as glucose, sucrose or lactose (Sugihara, Kline, and McCready, 1970). Maltose is the principal carbohydrate available in flour, approximately 5.5 percent based on dry weight, and is produced after the flour-water mixture is formulated, presumably by amylase action on free starch (Saunders, Ng, and Kline, 1972). This ability to ferment only maltose is particularly significant in light of the fact that the sourdough yeast is unable to utilize this sugar. Thus, the two microorganisms do not compete for the same carbohydrate source, a fact which has undoubtedly contributed to the stability of the system over such an extended period. The need for fresh yeast extractives (FYE) and stimulation of growth by CO_2 was predicted from the fact that the system contains an active yeast. Since flour contains about one to 1.5 percent of lipid, and two-thirds of the fatty acids in this lipid are unsaturated (Meeham, 1971), Tween 80 (polyoxyethylene sorbitan mono-oleate) was incorporated into the medium. The medium was adjusted to a pH below six since the normal habitat of these bacteria is limited to the narrow range of pH 3.8 to 4.5. The combination of nutritional factors necessary for successful isolation of the sourdough bacteria was thus found to include maltose, Tween 80, FYE, and an initial pH below six (Kline and Sugihara, 1971).

Four strains, designated B, C, L,and T (after the first letter of the bakery from which they were isolated) were obtained. Slight differences were observed among the four strains with regard to colony morphology, appearance in broth cultures, ability to adapt to glucose, and the proportion of end products produced, but all had the following characteristics in common: Gram-positive, non-motile rods, catalase negative, indifferent to oxygen, inhibited by sorbic acid; require FYE, Tween 80, and low pH for good growth, stimulated by CO_2, especially on agar media, and produce lactic acid, acetic acid and CO_2 as principle end products (Kline and Sugihara, 1971). Though their general characteristics tend to group them with the heterofermentative lactobacilli, their unique nutritional requirements place them outside the range of known Lactobacillus species. These authors therefore suggested the new species name Lactobacillus sanfrancisco for the sourdough bacteria.

In the natural sourdough environment, acetic acid generally comprises 20 to 30 percent of the total acidity produced. In pure broth cultures, however, the proportion of acetic acid was often found to be less than 10 percent and highly variable (Kline and Sugihara, 1971). This variation was found to depend almost entirely on the degree of aeration of the culture (Ng, 1972). Thus for strain L, the proportion of acetic acid produced was increased to 36 percent by aeration as compared to only about three percent under CO_2.

The heterofermenters L. brevis, L. buchneri, and L. fermenti responded to aeration in a manner similar to the sourdough bacteria, lending additional support to the preliminary tentative positioning of the sourdough bacteria as heterofermentative lactobacilli.

The classification of microorganisms based on their ability to utilize various substrates and upon the types of end products produced is, at best, a highly artificial system. Moreover, the phenotypic characters selected as taxonomically significant represent only a small fraction of the entire genome. Genetic distinctiveness among a group of closely related microorganisms can best be determined by comparing their genetic compositions and hybridization relationships. Analysis of sourdough bacterial deoxyribonucleic acid (DNA) showed an average composition of 38 to 39 percent guanine plus cytosine (GC) (Sriranganathan et al., 1973), a value which is substantially lower than that of known strains of heterofermentative lactobacilli. The genetic relatedness of bacteria to known species can be quantitatively determined by the use of the DNA-DNA hybridization technique, which offers a direct measure of the similarity or homology of the polynucleotide sequences in DNA molecules. Only bacteria having similar GC contents may be related to each other. A difference of only five to seven moles percent GC content significantly reduces the probability of two organisms having homology, indicating that the organisms in question belong to different species (Jones and Sneath,

1970). Since none of the known heterofermentative lactobacilli have a GC content comparable to those of sourdough bacteria, hybridization studies were undertaken with group I homofermentative organisms which are similar in GC content (Sriranganathan et al., 1973). The four sourdough isolates exhibited a high degree of homology among themselves (>88%), suggesting their taxonomic homogeneity, but they showed only 23 to 37 percent homology with four group I homofermentative lactobacilli. Genetic analyses therefore lended further support for consideration of the sourdough bacteria as a new heterofermentative species in the genus Lactobacillus.

Nutrition of Lactobacilli

The lactobacilli as a group are very fastidious organisms, generally requiring a variety of vitamins and assorted growth factors for rapid growth. Their exacting nutritional requirements presented a tantalizing puzzle in earlier studies because an unknown number of unidentified growth factors were required which could only be supplied as crude complex materials. Unidentified growth factors for lactic acid bacteria are of special biochemical and nutritional significance because these organisms have nutritional requirements remarkably similar to higher animals. Nutritional studies conducted on bacteria in defined and semidefined media led to the discovery of many new

growth factors, some of which were subsequently identified as vitamins required for other species, including man.

Vitamins

Biotin. The inability to synthesize biotin is common among the lactobacilli. Stokes, Larsen, and Gunness (1947a, b) found that biotin replaced the aspartic acid requirement of several lactobacilli and streptococci. Oxaloacetate also partially replaced biotin for growth of L. casei and L. plantarum, and it was suggested that biotin might be concerned with oxaloacetate formation. Aspartic acid could then result from transamination of the oxaloacetate. Williams and Fieger (1946) demonstrated that L. casei, which requires biotin, could be grown and maintained on an essentially biotin-free medium when oleic acid was present. Moreover, early work with biotin showed it to be involved in a rather bewildering variety of metabolic processes, some of them seemingly quite unrelated. The range of activities attributed to biotin include some aspects of the metabolism of carbohydrates, amino acids, proteins, nucleic acids or their components, and lipids. The process which is now best understood and supported by detailed evidence is carboxylation (Ochoa and Kaziro, 1965). For example, cells of L. plantarum grown in a low-biotin medium were almost completely unable to fix carbon dioxide;

cells grown in a medium with ample biotin fixed C^{14} from bicarbonate in the medium into cellular aspartic acid (Lardy, Potter, and Burris, 1949).

Folic Acid. The name folic acid was originally applied to a factor isolated from spinach by Mitchell, Snell, and Williams (1941) which was active in supporting growth of L. casei, L. delbrueckii, and Streptococcus faecalis R. In the earlier studies of this vitamin, a confusing variety of names was applied to it or closely related compounds as various investigators isolated it as an unidentified growth factor from different complex natural materials. Angier et al. (1946) showed folic acid from liver to consist of a pteridine, para-aminobenzoic acid, and glutamic acid, and suggested the name pteroylglutamic acid. Today, only the term folic acid is in common use, and is generally applied to any member of the group of related compounds. Tetrahydrofolate compounds play the role of co-enzyme in the transfer of one-carbon units. Specific reactions mediated include the following: (a) introduction of the C_2 and C_8 positions in purine biosynthesis; (b) formation of thymine by methylation of a pyrimidine intermediate; (c) conversion of glycine to serine; (d) formation of methionine by methylation of homocysteine; (e) introduction of the number 2 carbon of the imidazole ring of histidine; (f) choline formation by methylation of dimethyaminoethanol;

(g) methylation of nicotinamide to N-methylnicotinamide (Koser, 1968).

Para-aminobenzoic Acid. This compound is a part of the folic acid molecule, but its activity as a separate substance has also attracted attention. Repeated evidence has shown that para-aminobenzoic acid (PABA) is utilized by L. plantarum for the synthesis of folic acid and related compounds (Sarett, 1947; Nimmo-Smith, Lascelles, and Woods, 1948; Hendlin, Koditschek, and Soars, 1953). However, most lactobacilli grow equally well without the addition of PABA provided the medium contains other necessary nutrients. Contrary to some earlier investigators, Rogosa, Franklin, and Perry (1961) concluded that PABA is not essential or stimulatory for any species of Lactobacillus.

Nicotinic Acid. Most strains of lactobacilli require some form of nicotinic acid for growth. Twenty-six oral lactobacilli examined by Koser and Fischer(1950) exhibited a definite requirement for this compound, as did several hundred lactobacilli tested by Rogosa et al. (1953). As a group, the lactobacilli respond about equally well to nicotinic acid and nicotinamide; many also respond to the co-enzyme form of nicotinic acid, nicotinamide adenine dinucleotide. The co-enzyme forms of nicotinamide occur in all living cells and play an

essential part in electron transport in the process of biological oxidation.

Pantothenic Acid. Concentrated from liver by Snell, Strong, and Peterson (1937), who termed it L fraction, pantothenic acid is a definite requirement for practically all lactobacilli (Cheldelin, Hoag, and Sarett, 1945; Rogosa et al., 1953). For some strains, the active form is pantethine or pantetheine, which represent the oxidized and reduced forms of a combination of pantothenic acid with β-mercaptoethylamine or cysteamine. Before the structures were known, these compounds and related substances were referred to as the "Lactobacillus bulgaricus factor" or LBF. LBF was found by Craig and Snell (1951) to be 20 to 400 times as active as pantothenic acid for some of the more fastidious lactobacilli. The functional forms of pantothenic acid are as components of Coenzyme A (CoA) and of 4'-phosphopantetheine. As an acetyl or acyl carrier, CoA plays a part in a variety of metabolic activities; for example, the formation of citric acid (six carbons) from oxalacetic acid (four carbons). The pantothenic acid conjugate, 4'-phosphopantetheine, has been identified as a prosthetic group of an acyl carrier protein which is involved in fatty acid synthesis (Alberts, Majerus, and Vagelos, 1965; Wakil, 1965).

Pyridoxine. The term vitamin B_6 is commonly used as a class name for pyridoxine and several chemically related compounds. Pyridoxine was demonstrated to exhibit growth-promoting activity for some lactobacilli (Snell and Peterson, 1940), and the activity was greatly enhanced by heat sterilization of the medium (Snell, Guirard, and Williams, 1942). This enhancement was found to be caused by the formation of pyridoxamine and pyridoxal, substances which are much more active for most lactobacilli (Snell and Rannefeld, 1945). Some lactobacilli require a phosphorylated form of vitamin B_6, pyridoxamine phosphate or pyridoxal phosphate (McNutt and Snell, 1950). A role for vitamin B_6 in amino acid metabolism was indicated by subsequent studies in which a number of lactobacilli were found not to require vitamin B_6 compounds for growth in casein digest or complete amino acid synthetic media. Further studies showed that pyridoxine and pyridoxamine are converted to pyridoxal-5-phosphate, which serves as a coenzyme in a wide variety of enzymatically catalyzed reactions of amino compounds, chiefly α-amino acids. Among the reactions are those involving transamination, racemization, and decarboxylation (Snell, 1958).

Riboflavin. In general, practically all homofermentative lactobacilli require riboflavin, while heterofermentative lactobacilli do not require it, but require thiamine instead (Rogosa, Franklin,

and Perry, 1961). Free riboflavin has no known coenzymatic function, but flavoprotein enzymes occupy a position in the chain of respiratory enzymes between nicotinamide adenine nucleotide enzymes and the cytochromes. They serve to transfer hydrogens or electrons from reduced nicotinamide adenine dinucleotide or its related triphosphate form to cytochrome. In the case of several lactobacilli, the transfer is thought to be directly to molecular oxygen (Strittmatter, 1959).

Thiamine. Thiamine is usually required by heterofermentative but not by homofermentative lactobacilli. The vitamin consists of two moeties, a pyrimidine ring and a thiazole ring. Some organisms, such as L. viridescens, require intact thiamine and are unable to make use of the two components unless both are supplied (Deibel, Evans, and Niven, 1957). On the other hand, the thiazole in the absence of added pyrimidine supported growth of eight out of ten heterofermentative lactobacilli, most of them L. fermenti, examined by Koser and Thomas (1953). The active form of thiamine is the pyrophosphoric ester known as co-carboxylase. One of the more familiar reactions catalyzed by enzymes containing thiamine pyrophosphate is the decarboxylation of α-keto acids, such as pyruvic acid, with the formation of acetaldehyde and carbon dioxide.

Vitamin B-12. A vitamin B_{12} requirement for certain fastidious lactobacilli was first observed by Shorb (1948), who reported that this compound could replace purified liver extracts in the nutrition of some strains of L. lactis and L. leichmannii. This vitamin can often be replaced by purine or pyrimidine deoxyribosides, but much larger amounts of these compounds are necessary for equivalent growth (Kitay, McNutt, and Snell, 1950). Vitamin B_{12} requirements are often lowered in the presence of reducing agents or reduced oxygen tension. Greene, Brook, and McCormack (1949) reported that L. lactis does not require B_{12} for anaerobic growth.

Vitamin B_{12} contains one atom of cobalt that is bound by coordinate linkages to the four nitrogen atoms of a partially hydrogenated tetrapyrrole, to a cyanide group and to a nucleotide. Cobamide coenzymes, the active forms of B_{12}, differ from one another by substitutions at the nucleotide moiety, but all differ from the vitamin by the absence of cyanide and its replacement by an adenine nucleoside (Weissbach, Peterkofsky, and Barker, 1965).

Vitamin B_{12} has been implicated in a wide variety of reactions. Among the enzymes dependent upon a cobamide coenzyme are methylmalonyl CoA isomerase, glutamate isomerase, and glycol dehydrase (Jaenicke, 1964). Blakley (1965) suggested that it is involved in deoxyribose formation in L. leichmannii. Dubnoff and Bartron (1956) reported that vitamin B_{12} apparently plays a part in maintaining

sulfhydryl groups in the reduced state. This could account for the decreased vitamin B_{12} requirement of lactobacilli grown anaerobically or in the presence of reducing agents, but the importance of this role is reduced in view of the small amount of B_{12} available in the cell.

Nucleic Acid Components

A large variety of nucleic acid derivatives have been shown to play a role in the nutrition of lactobacilli. The effect of these compounds is usually that of a growth stimulant rather than an absolute requirement. Snell and Mitchell (1941) found no definite requirement for nucleic acid bases in the growth of L. pentosus and L. plantarum, but adenine markedly stimulated growth. Ikawa and O'Barr (1956) found adenosine to be as good a stimulant for L. delbruckii as was yeast extract. Cogan, Gilliland, and Speck (1968) reported that both of these compounds were stimulatory for L. bulgaricus. The requirement for purines and pyrimidines may vary with the composition of the culture medium. The literature abounds with examples of complex nutritional interrelationships. Purines exerted only a slight stimulatory effect for a strain of L. casei grown in the presence of folic acid, but when thymine was substituted for the vitamin, purines were required (Hitchings et al., 1950).

Similarly, Soska (1966) found that a strain of L. acidophilus could be grown with thymine in the place of folic acid in a chemically defined medium. Under these conditions, thymine was required, as well as a deoxyriboside, purines, pyrimidines, and a number of vitamins and amino acids. Furthermore, utilization of thymine, but not of thymidine, was inhibited by adenine and adenosine. An inhibition of growth of L. bulgaricus GS was observed with deoxyadenylic acid and deoxyguanylic acid which could be reversed by two unidentified growth factors in liver extract (Morris and Williams, 1965). Such examples of the complicated interplay of different nucleic acid derivatives make it clear that a delicate balance often exists among the components of culture media.

The response of lactobacilli as a group to the purines and pyrimidines is varied, and often the stimulatory effect is not specific to any one compound, but may be shown by several. For some of the more fastidious lactobacilli, the deoxyriboside derivative may support growth while the free base does not. As mentioned previously, the deoxyribosides and their phosphorylated derivatives are often effective in replacing vitamin B_{12}. Lampen and Wang (1952) observed strong nucleoside hydrolase activity in L. pentosus, which may serve as an important salvage pathway for purine and pyrimidine nucleosides.

Peptides

Peptides have long been known to stimulate the growth of bacteria, especially the lactic acid bacteria. In this connection, a substantial literature accumulated concerning the nature of "strepogenin," a factor present in partial hydrolysates of proteins that stimulates growth of L. casei in defined media (Sprince and Woolley, 1944). It was generally believed that strepogenin was a peptide, or at least peptide-like in nature, but later investigation showed little correlation between the biological activity and the sequence of amino acid residues or the exact nature of these residues (Woolley and Merrifield, 1958). Peptides or enzymatic digests of protein were found to be stimulatory or required for growth of many fastidious lactobacilli. Among them were several strains of L. bulgaricus, L. lactis, and L. helveticus (Kitay and Snell, 1950), L. bifidus (Gyllenberg, Rossander, and Roine, 1953), and L. bifidus var. pennsylvanicus (Györgi and Rose, 1955). In most cases, peptides apparently serve only to supply a limiting amino acid, the free form of which is not utilized efficiently for growth, rather than to supply a unique sequence of amino acids. Kihara and Snell (1960a) outlined several conditions which would reduce the availability of an essential amino acid for bacterial growth, thereby enhancing the ability of appropriate peptides to promote growth: (a) assimilation

of a free amino acid, but not of its peptides, may be inhibited by the presence of an antagonistic amino acid; (b) a free essential amino acid, but not appropriate peptides of it, may be partially destroyed in side reactions by the organism before it can be utilized for growth; and (c), absorption of a given essential amino acid may be less efficient than that of its peptides for reasons other than the presence of an antagonistic amino acid; e.g., the efficient absorption of the free amino acid but not of appropriate peptides may require increased levels of structurally unrelated amino acids or other nutrients in the medium. These authors were of the opinion that peptides play no special role in metabolism, but rather that their addition to culture media results in a better adjustment to the nutritional needs and peculiarities of the particular organism by alleviating the competition of individual amino acids in penetrating the cellular barriers. Such conclusions, however, have been reached with selected strains of lactic acid bacteria and may not necessarily be a general occurrence among the many organisms that respond in growth to peptides. Fox (1961) demonstrated that streptococcal proteinase synthesis requires exogenous peptides, and suggested that peptides may participate in a more direct manner in protein synthesis. Confirmation of such participation awaits demonstration of the appearance of labeled peptides in protein synthesized de novo with the labeled precursors in their original sequence.

Inorganic Nutrition

Knowledge of inorganic nutrition is relatively advanced for organisms with simple organic requirements (Hutner, 1972). Studies on inorganic requirements of lactic acid bacteria are made difficult, however, by the complexity of the nutritive requirements of these organisms. Complex media suitable for their growth usually contain, as contaminants, sufficient essential mineral elements to permit limited or extensive growth even though none of the mineral is added. Furthermore, the presence of large amounts of organic material is not compatible with many of the procedures used in other investigations for removal of traces of inorganic ions.

Interest in inorganic nutrition was stimulated by observations of the presence of heat resistant growth factors in complex materials such as tomato juice, cabbage, and asparagus (Metcalf, Hucker, and Carpenter, 1946; Snell and Lewis, 1953). Stamer, Albury, and Pederson (1964) demonstrated that manganese can account for the biological activity of tomato juice in the nutrition of 63 strains of lactic acid bacteria tested. Some organisms exhibit a linear growth response to certain ions, which can be made the basis for a microbiological assay. Lactobacillus casei has been suggested for use in a microbiological assay for potassium (Rogosa, 1944), and L. arabinosus for the determination of manganese (Bentley, Snell, and

Phillips, 1947). MacLeod and Snell (1947) determined the essentiality of manganese, potassium, and phosphate for Leuconostoc mesenteroides, Streptococcus faecalis, and a number of lactobacilli in a synthetic medium. Magnesium stimulated growth of these organisms but was not essential. Iron is generally included in synthetic media for lactic bacteria (Henderson and Snell, 1948), but omission of iron did not deleteriously affect the growth of L. arabinosus, L. casei, L. delbrueckii, L. fermenti, or L. pentosus (MacLeod and Snell, 1947). Since these organisms grow anaerobically, do not contain cytochrome, and are catalase negative, one would expect that if iron is required at all, it would be required only in very small amounts.

Since it is extremely difficult to control trace element contamination from glassware and reagent grade chemicals, demonstrated mineral requirements probably only represent those inorganic ions which are required in the largest amounts for growth. Others will undoubtedly be found to be required or stimulatory. Owen and Pringle (1970), for example, found evidence of a molybdenum requirement for L. casei.

Yeast Extractives

Little more than 20 percent of the yeast produced by batch fermentation methods in the brewing industry is required for seeding

subsequent batches (Acraman, 1966); the excess yeast is the basic raw material for the yeast extract industry. Yeast extract consists of the proteins, nucleic acids, carbohydrates, and other intracellular material extracted from yeast cells by autolysis. The term autolysis actually refers to "self-destruction" of the yeast cells by intracellular enzymes, but it has been broadened to include a whole range of artificial methods by which the intracellular material is rendered soluble. Most of the yeast extract produced is used as food additives in a variety of products including meat pie fillings, gravies, soups, and sauces. In addition to these uses, bacteriologists quickly recognized the value of yeast extract as an excellent nutrient source in bacteriological culture media (Ayers and Rupp, 1920).

Early studies in microbial nutrition readily established that something more than an organic nitrogen compound, a carbohydrate, and inorganic salts was required by many microorganisms. Although certain microorganisms could be cultivated in quite simple nutrient solutions, most required complex infusions of natural products. Wildiers (1901) reported that a strain of Saccharomyces cerevisiae which grew poorly in a solution of sucrose, ammonium chloride, and salts grew readily when a filtered extract of yeast cells was added to the solution. This extract apparently contained an unknown growth-promoting factor which he called "bios". The nature of bios remained obscure for several decades, but it came to be regarded as a complex

mixture of substances rather than a single compound. There was much speculation about the relationship of microbial growth factors and the vitamins of human nutrition, but the chemical complexity of infusions and the limitations of analytical methods available at the time tended to discourage earlier investigations along this line. Lucas (1924) separated bios into two fractions designated bios I and bios II, and Eastcott (1928) reported that the activity of bios I was due to inositol. Bios II was further fractionated into a number of biologically active components including substances identified as panthothenic acid (Williams and Major, 1940) and biotin (Kögl and Tönnis, 1936). Knight (1937a,b) reported that a concentrate of yeast, containing growth-promoting activity for Staphylococcus aureus, could be replaced by nicotinic acid and thiamine. Thus ensued a gradual merging of bacterial growth factors and vitamins.

Yeast extract is a good source of vitamins of the B complex group, except for B_1, which rapidly diminishes during storage (Pyke, 1958). In addition, it serves as an excellent source of growth promoting substances other than vitamins, such as ribonucleotides and deoxyribonucleotides found to be important in the nutrition of a strain of Lactobacillus acidophilus (Eyssen et al., 1965), unidentified growth factors for L. bulgaricus GS (Weinman, Morris, and Williams, 1964; Lin and Williams, 1966), and trace elements (Grant and Pramer, 1962).

Details of industrial processes for production of yeast extracts are not readily available, and in many cases parts of the processes are regarded as highly secret. Some of the process routes which can be adopted for extract production are shown in Figure 1.

Yeast cytoplasm contains a highly organized system of complex chemical components. When the osmotic integrity of the cell membrane is destroyed at death, low molecular weight soluble compounds are able to diffuse out, but insoluble high molecular weight material is retained inside the cell. Commercial methods for the production of yeast extractives depend on the breakdown of insoluble complex materials to soluble units in order to obtain a satisfactory yield. In general, these solubilization techniques can be classified as autolytic, plasmolytic, and hydrolytic processes.

The autolytic process is based on the careful application of sufficient heat to kill the yeast cells without inactivating enzymes important for autolysis. With delicate control mechanisms no longer functioning, the enzymes indiscriminately attack their substrates, breaking complex cell components into smaller units which can be readily washed from the cells to give a high yield yeast extract. The factors influencing the rate and extent of autolysis, and the chemical and cytological change involved, have been extensively studied (Josyln, 1955; Joslyn and Vosti, 1955), but the biochemical basis of

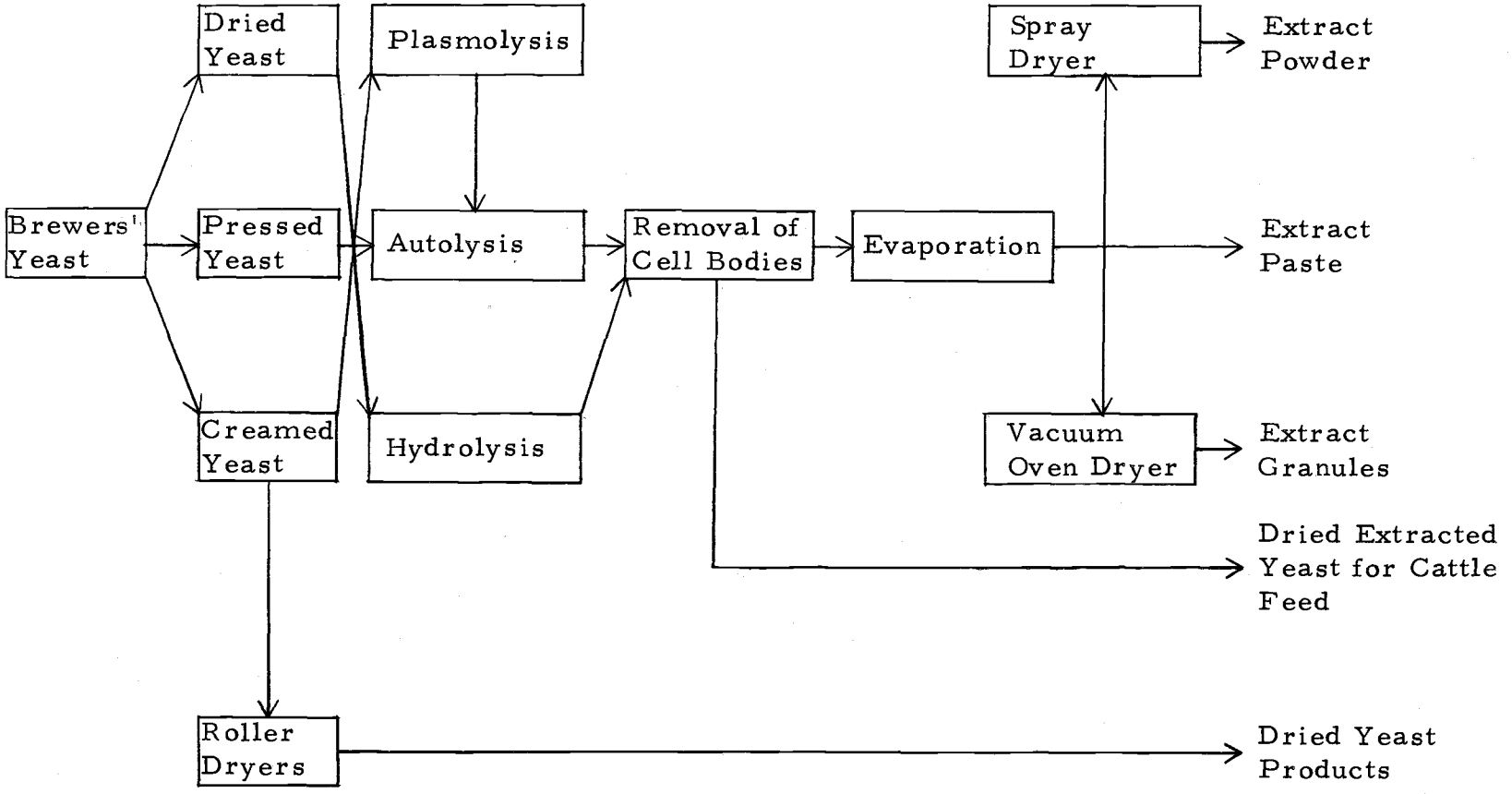

Figure 1. Industrial process routes in the manufacture of yeast extractives (Adapted from Acraman, 1966).

autolysis is not yet completely understood. Maddox and Hough (1970) have isolated and characterized four distinct proteolytic enzymes from autolyzing brewers' yeast. It has been suggested that these enzymes are released by starved, living cells during the course of autolysis in response to the release of protein by dead cells (Hough and Maddox, 1970). Some of the proteolytic activity in the medium will be derived from dead cells, and hydrolytic enzymes, other than proteases, liberated at the same time will degrade macromolecules such as nucleic acids. This process proceeds within the yeast mass until all the cells die and complete autolysis occurs.

Plasmolysis is essentially a method of rapidly instituting autolysis. Increasing the osmolarity outside the cell by adding inorganic or organic solutes to the suspending menstruum causes the cytoplasm to shrink away from the cell wall as the cell loses water into the surroundings. Under these conditions, the cell rapidly dies and autolysis follows. Commercial processes generally depend on the addition of sodium chloride (Acraman, 1966). This method has the advantages of rapid initiation of autolysis and a certain degree of bacterial growth retardation by the presence of salt, but the final product contains an undesirably high salt concentration. In order to avoid this saltiness, a number of alternative plasmolyzing agents such as amyl acetate and ethyl acetate have been suggested (Pyke, 1958).

Hydrolytic methods generally utilize hydrochloric acid under various conditions of concentration, temperature, and pressure (Acraman, 1966). Under these conditions, it is possible to solubilize a greater proportion of the cell contents than by autolytic or plasmolytic processes, but much of the increase results from the breakdown of carbohydrate rather than protein. Moreover, the advantage of high yields is counterbalanced by the introduction of high salt concentrations into the finished product, the presence of high carbohydrate content, and the loss of certain amino acids and vitamins, generally making hydrolysis the least desirable production process.

METHODS AND MATERIALS

Microorganisms

Four strains of sourdough bacteria, designated as B, C, L, and T after the first letter of the bakery from which they were isolated, and Saccharomyces exiguus, were obtained from the USDA Western Regional Research Laboratory, Albany, California.

Saccharomyces cerevisiae was isolated from commercial compressed bakers' yeast (Fleischmann's, Standard Brands, Inc.). Saccharomyces carlsbergensis was obtained from the culture collection of the Department of Microbiology, Oregon State University.

Bacterial cultures were maintained in sourdough broth by transferring weekly and storing at 4 C following growth for 24 hr at 31.5 C. Inocula for test cultures were prepared by sedimenting cells by centrifugation and resuspending in a volume of sterile physiological saline equal to that of the original sample. Yeast cultures were maintained at 4 C on slants of yeast medium (YM agar).

In the interests of consistency and simplicity, the results presented in this thesis are those obtained with strain T. Where significant strain differences became apparent, results for all four strains are shown.

Culture Media

The composition of sourdough broth (SDB) shown in Table 1 is essentially that described by Kline and Sugihara (1971). SD agar was prepared by incorporating 1.5% agar into the medium. A complex basal medium (CBM), the composition of which is shown in Table 2, was used in studying the effects of yeast extractives and metal ions on the growth of sourdough bacteria, but had the disadvantages of an ill-defined chemical composition and the ability to support limited growth of the microorganisms when unsupplemented. A semi-synthetic basal medium (SSBM) (Table 3) was developed which supported little or no growth of the sourdough bacteria when unsupplemented. Moreover, the defined nature of the components made it possible to determine vitamin and purine-pyrimidine requirements when supplemented with a suitable purified fraction of fresh yeast extractives (FYE). When supplemented with FYE or active fractions of FYE, SSBM supported approximately the same amount of growth as SDB. YM broth for growth of yeasts contained 1.0% glucose, 0.3% malt extract, 0.5% peptone, and 0.3% yeast extract. YM agar was prepared by adding 1.5% agar to the formulation.

Table 1. Composition of sourdough broth (SDB).

Component	Percent by Weight
Maltose	2.0
Trypticase	0.6
Difco Yeast Extract	0.3
Fresh Yeast Extractives	0.5
Tween 80	0.03
pH 6.0	

Table 2. Composition of complex basal medium (CBM).

Component	Percent by Weight
Maltose	2.0
Trypticase	0.6
Difco Yeast Extract	0.5
Tween 80	0.03
pH 6.0	

Table 3. Composition of semi-synthetic basal medium (SSBM).

Component	Percent by Weight
Maltose	2.0
Vitamin-free casamino acids	1.0
L-tryptophan	0.5
Tween 80	0.03
Sodium acetate	0.01
Sodium citrate	1.0
NH_4Cl	0.3
K_2HPO_4	0.5
$MgSO_4$	0.08
NaCl	0.004
$FeSO_4 \cdot 7H_2O$	0.004
$MnSO_4 \cdot 4H_2O$	0.016
Thiamine	0.0005
Riboflavin	0.0005
Nicotinic acid	0.0005
Calcium pantothenate	0.0005
Pyridoxamine	0.0005
Para-aminobenzoic acid	0.0001
Biotin	0.00005
Folic acid	0.00005
Adenine sulfate	0.005
Guanine HCl	0.005
Uracil	0.005
Xanthine	0.005
pH 6.0	

Measurement of Growth

Growth in broth cultures was quantitated with the use of a Bausch and Lomb Spectronic 20 spectrophotometer at 600 nm. Cultures were diluted when necessary to stay within an absorbance range of approximately 0 to 0.4, the range within which Beer's law is followed. Growth curves were prepared by removing aliquots of a broth culture at intervals and plating dilutions on SD agar. Following incubation at 31.5 C for 48 hr in an atmosphere containing approximately 20% CO_2, plate counts were determined using standard methods. Generation times were determined by use of the formula:

$$G = \frac{0.3010 \times t}{\log b - \log a}$$

where a and b are the number of bacteria present at two sampling times during logarithmic growth, and t is the elapsed time between samplings.

Preparation and Source of Yeast Extractives

Fresh yeast extractives (FYE). Commercial compressed bakers' yeast (Fleischmann's, Standard Brands, Inc., New York) was obtained from local bakeries as one pound bricks. The yeast was blended at low speed with sufficient distilled water to form a 20% suspension, and autoclaved for 30 min at 121 C. Solids were allowed to settle overnight at 4 C, and the cloudy supernatant was clarified

by centrifugation. The resulting clear solution was lyophilized and stored at 4 C.

Commercial yeast extractives and other complex materials, obtained from the following sources, were dissolved in distilled water to form a 5% solution, filtered if necessary, lyophilized, and stored at 4 C:

Difco yeast extract (DYE), Difco Laboratories, Detroit, Michigan. Lot number 496088 was used throughout this study.

Red Star autolyzed yeast (RS), Universal Foods Corporation, Milwaukee, Wisconsin.

Autolyzed yeast paste #75-NAS, A. E. Staley Manufacturing Co., Decatur, Illinois.

Maggi autolyzed yeast (MSP), special light powder, lot #16, The Nestle Co., White Plains, New York.

Universal Foods Corporation (UFC), dry yeast powder, Milwaukee, Wisconsin.

Corn steepwater (CS), Corn Industrial, a division of CPC International, Englewood Cliffs, New Jersey.

Vanco fish peptone (FP), lab. control no. 70-3351, Van Camp Laboratories, Terminal Island, California.

Preparation of FYE from Pure Cultures

Yeast extractives were prepared from pure cultures of S. cerevisiae, S. exiguus, and S. carlsbergensis. Ten liters of YM broth was placed into a 14 liter fermenter vessel (Fermentation Design, Inc.) and autoclaved for 30 min at 121 C. After cooling to room temperature, the medium was inoculated with 200 ml of a 24 hr yeast culture and incubated under the following conditions:

Agitation: 200 RPM

Aeration: Sterile air, 4 liters/min

Temperature: 25 C

Foam Control: Dow Corning Y-30 emulsion

After growth for 72 hr, the cells were harvested using a Sharples continuous-flow centrifuge, and FYE was prepared as previously described.

Effect of Temperature and pH During Preparation of FYE

To test the effect of temperature during FYE production, extractives were prepared as previously described, however, instead of being autoclaved, the yeast suspension was subjected to various controlled temperatures in a water bath for 30 min and immediately cooled to 4 C.

The effect of pH was studied by heating the yeast suspension to boiling to kill the cells, cooling to room temperature, and adjusting the pH to the desired level with 1 N HCl or NaOH. The suspension was then autoclaved for 30 min at 121 C, cooled, adjusted to pH 6.0, and treated further as previously described for FYE.

Autolysis vs. Hot-Water Extraction

A 50% suspension of compressed bakers' yeast in distilled water was allowed to autolyze for 24 hr in a controlled temperature water bath. The suspension was then centrifuged, and the clear supernatant (autolysate) was lyophilized and stored at 4 C. The pellet was resuspended in distilled water to form a 20% suspension, and further treated as in the production of FYE.

Growth Stimulation by Divalent Cations

The effect of added divalent cations was studied in CB medium. Sulfate salts of the metals were added to the medium in amounts calculated to supply the cations at concentrations of 10 μg/ml. Growth was measured after 24 hr of incubation at 31.5 C.

Fractionation of FYE

A flow scheme of the procedures used for the isolation of active fractions from FYE is shown in Figure 2.

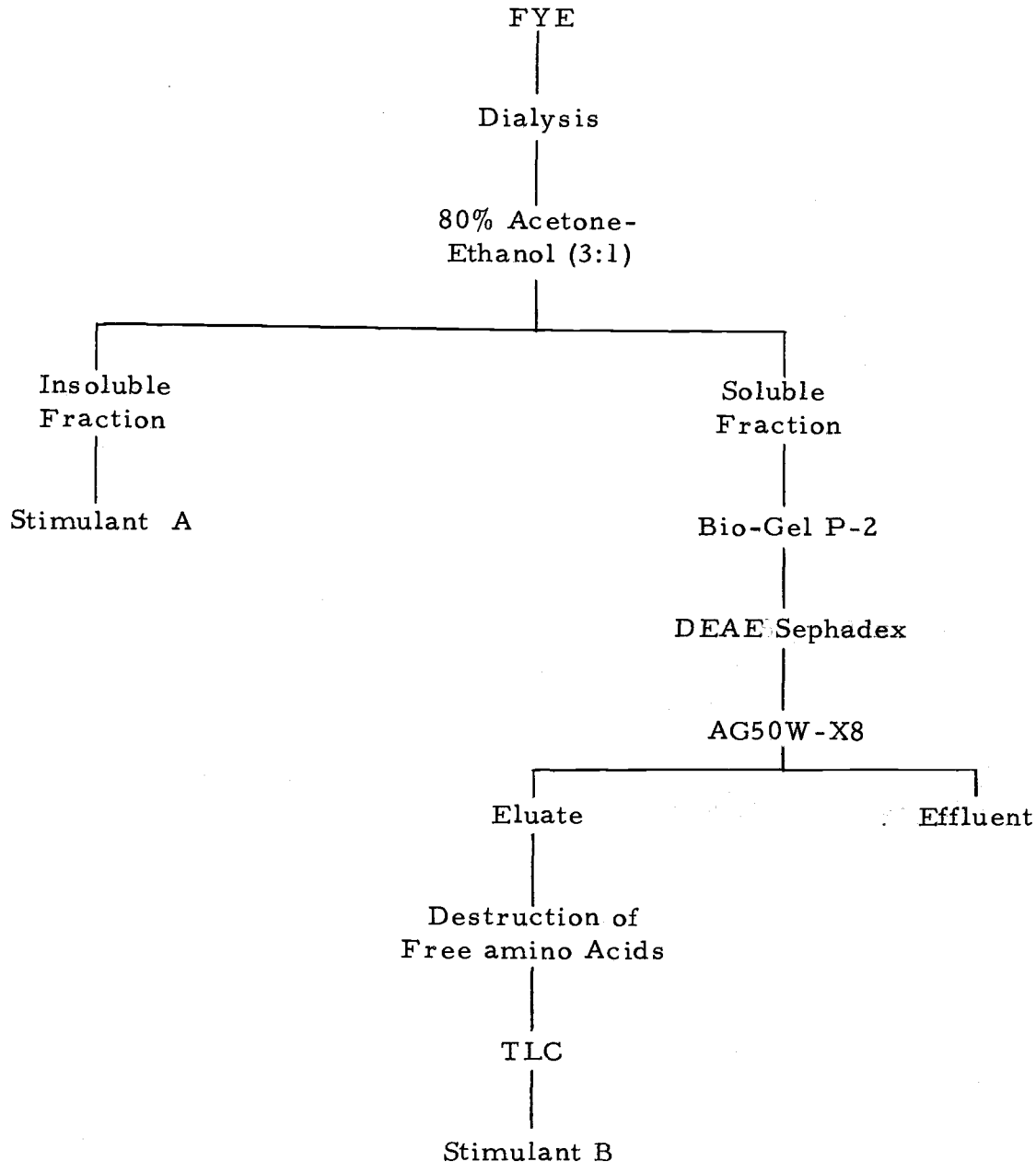

Figure 2. Flow scheme of techniques used for the isolation of growth factors for sourdough bacteria from FYE.

Dialysis

Ten liters of a 20% suspension of compressed bakers' yeast in distilled water was autoclaved for 30 min at 121 C and cooled to 4 C. Dialysis bags (Union Carbide, 11 cm flat width) containing approximately 500 ml of distilled water were suspended in the slurry, and dialysis was allowed to continue for 72 hr at 4 C with continuous stirring. At the end of this period, the dialysate was removed from the bags, lyophilized, and stored at 4 C.

Acetone - Ethanol Fractionation

To 100 ml of 5% FYE dialysate in distilled water was added 400 ml of a 3 to 1 mixture of acetone and ethanol. The mixture was held at -5 C for 4 hr, then centrifuged in the cold to sediment the precipitate. The clear supernatant (FYES) was decanted, reduced under vacuum to dryness, and dissolved in 100 ml of distilled water. This solution, which formed no further precipitate when treated with 10% trichloroacetic acid, was lyophilized and stored at 4 C. The pellet (FYEP) was reduced to dryness under vacuum, dissolved in 100 ml of distilled water, and reprecipitated with acetone and ethanol. After drying, the precipitate was dissolved in water, lyophilized, and stored at 4 C.

Gel Filtration

Fifty milligrams of FYES contained in 0.5 ml of distilled water was applied to a 1.3 by 21 cm column of Bio-Gel P-2, 100-200 mesh (Bio-Rad Laboratories), equilibrated at room temperature with distilled water. The column was eluted with distilled water at the rate of 0.5 ml/min, and 2.5 ml fractions were obtained using an UltroRac model 7000 fraction collector (LKB Instruments, Inc.). Fractions were pooled according to their absorbance at 260 nm as measured with a Beckman model DU-2 spectrophotometer, lyophilized, and assayed for growth promoting activity in SSBM.

Anion Exchange Chromatography

DEAE Sephadex A-25-120 was obtained from Sigma Chemical Co. and converted to the acetate form by swelling the dry beads in 2 M sodium acetate, pH 4.5, packing into a column, and washing with the same buffer until free of chloride ion. The ion-exchanger was then transferred to a beaker and suspended in 0.5 M triethylammonium acetate (TEAA) buffer, pH 4.7, (starting buffer), prepared as described by Caldwell (1969). After several washings by decantation with TEAA buffer, the ion-exchanger was packed into a column (1.3 by 25 cm) and equilibrated at room temperature with starting buffer. The lyophilized active fraction from gel filtration was dissolved in 0.5 ml of starting buffer and applied to the column, which

was eluted with a gradient of 0.5 to 1.5 M TEAA buffer, pH 4.7. Fractions of 2.5 ml were collected at a flow rate of 0.5 ml/min. Fractions were pooled according to their absorbance at 260 nm and by the relative color intensity of the ninhydrin test, lyophilized, and tested for growth promoting activity in SSBM.

Cation Exchange Chromatography

The cation exchange resin AG50W-X8 (H^+ form) was obtained from Bio-Rad Laboratories. After washing with 0.3 N HCl, the resin was packed into a column (1.3 by 20 cm) and washed with distilled water until the eluate gave a negative test for chloride ion. The active fraction obtained from anion exchange chromatography was dissolved in 2 ml of distilled water, applied to the column and washed through with 200 ml of distilled water, then eluted with 200 ml of 2 N NH_4OH. Both the effluent and eluate were lyophilized and tested for growth promoting activity in SSBM.

Destruction of Free Amino Acids

The procedure of Markovitz and Steinberg (1957) as modified by Koburger, Speck, and Aurand (1963) was used to remove free amino acids from biologically active components. The active fraction following cation exchange chromatography was dissolved in 5 ml of citrate buffer, pH 2.5, 100 mg of ninhydrin was added, and the mixture

was heated for 10 min in a boiling water bath. The reaction mixture was immediately cooled, filtered, extracted three times with 50 ml volumes of diethyl ether to remove unreacted ninhydrin, and lyophilized.

Thin Layer Chromatography

The active fraction was finally isolated on 0.5 mm layers of silica gel G. The material remaining after destruction of free amino acids was dissolved in 1 ml of distilled water and applied as a compact band on 20 by 20 cm plates. Following development with the organic phase of an n-butanol - acetic acid - water (5 : 1 : 4) mixture, a narrow band at the edge of the plate was sprayed with ninhydrin (0.2% ninhydrin and 0.5% acetic acid in n-butanol) to locate alpha-amino compounds on the chromatogram. The remainder of the band was scraped off the plate and eluted with distilled water. The active component was tested for growth promoting activity in SSBM and identified by amino acid analysis.

Chemical Analyses

DNA and RNA

To prepare FYE for determination of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) contents, 100 mg was suspended

in 20 ml of 5% trichloroacetic acid and heated at 80 C for 15 min. After removal of protein by centrifugation, DNA was determined by the method of Ceriotti (1952) using calf thymus DNA as standard. The method of Dische and Borenfreund (1957) was used for the determination of RNA, using yeast RNA as standard.

Protein

The protein content of FYE was determined by the biuret method (Gornall, Bardawill, and David, 1948), using bovine albumin as the standard. Alpha-amino compound content as a criterion for pooling fractions eluted from columns was determined by the ninhydrin tube test as described by Clark (1964).

Carbohydrate

Total carbohydrate content of FYE was measured by the anthrone method (Seifter, et al., 1950) using glucose as standard. The carbohydrate content of column eluates was determined in a similar manner, but was not quantitated.

Amino Acids

The amino acid content of unhydrolyzed and hydrolyzed stimulatory fractions isolated from FYE was determined by the method of

Moore and Stein (1963) with the aid of a Beckman model 120B amino acid analyzer, and by thin layer chromatography on 0.25 mm layers of cellulose MN 300. Plates were developed with phenol-water (75:25) using 0.1% solutions of known amino acids as standards.

Manganese

A technique based on the data of Gentry and Sherrington (1950), which depends upon the solubility of the 8-hydroxyquinolinates of manganese in chloroform, was used to remove this metal from complex culture media. One hundred ml of culture medium was adjusted to pH 8.0 with 2 N NH_4OH and extracted three times with 20 ml portions of 1% 8-hydroxyquinoline in chloroform. It was then extracted once with chloroform to remove traces of 8-hydroxyquinoline and centrifuged to break the chloroform-water emulsion. The pH was adjusted to 6.0 with 2 N HCl and the medium was heated to boiling to remove traces of chloroform.

Samples of yeast extractives and lyophilized culture media were ashed at 650 C in a Thermolyne type 1500 muffle furnace. The ash was dissolved in a small amount of concentrated HCl and evaporated to dryness over a boiling water bath. After dissolving the residue in distilled water, the manganese content was determined by the method of Cornfield and Pollard (1950).

Determination of Vitamin Requirements

Vitamin and purine-pyrimidine requirements of the sourdough bacteria were determined in SSBM supplemented with the active fraction of FYE purified through the cation exchange step. Lots of SSBM which were complete except for one vitamin were prepared, distributed in 2 ml quantities, and autoclaved for 5 min at 121 C. Cells for inoculum were washed twice with sterile physiological saline and made to the original volume from which one drop was added to each tube. The degree of growth in comparison to growth in the complete medium was recorded after 24 and 48 hr at 31.5 C. Cultures showing less growth than occurred in the complete medium were transferred to fresh media (without a particular vitamin) to determine whether the vitamin in question was required, not required, or stimulatory.

RESULTS

Growth of Sourdough Bacteria

A typical growth curve of sourdough bacteria in SD broth at 31.5 C is shown in Figure 3. Maximum cell numbers were obtained after 20 to 22 hr of incubation, with a minimum generation time of about 51 min. From the data of Kline, Sugihara, and McCready (1970), a generation time of approximately 99 min can be calculated for sourdough bacteria during sourdough bread proofing.

Growth of sourdough bacteria in the presence of FYE and commercial yeast extractives is shown in Figure 4. Various degrees of growth promoting activity were exhibited by the commercial extractives, but FYE was clearly superior to all commercial extractives tested. Increasing the concentrations of extractives in the medium up to 2% generally increased the amount of growth obtained. Red Star autolyzed yeast became slightly inhibitory, and Difco yeast extract markedly inhibitory, however, at concentrations exceeding 1.5%. In no case did concentrations exceeding 2% increase growth significantly.

Since most commercial yeast extractives are prepared from brewers' yeast, it was conceivable that the superior qualities of FYE were due to the bakers' yeast from which it is routinely prepared.

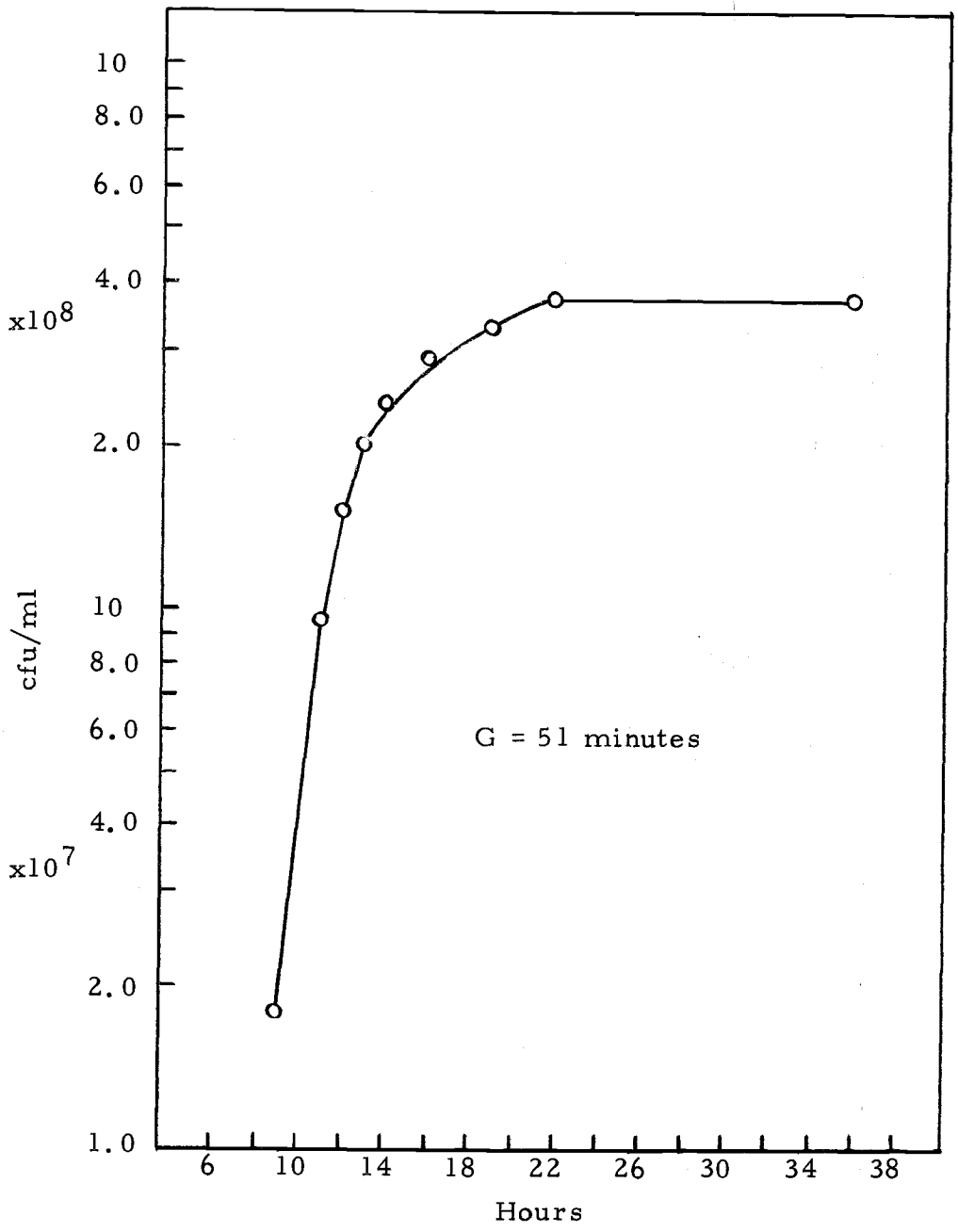

Figure 3. Growth curve of sourdough bacteria in SD broth.

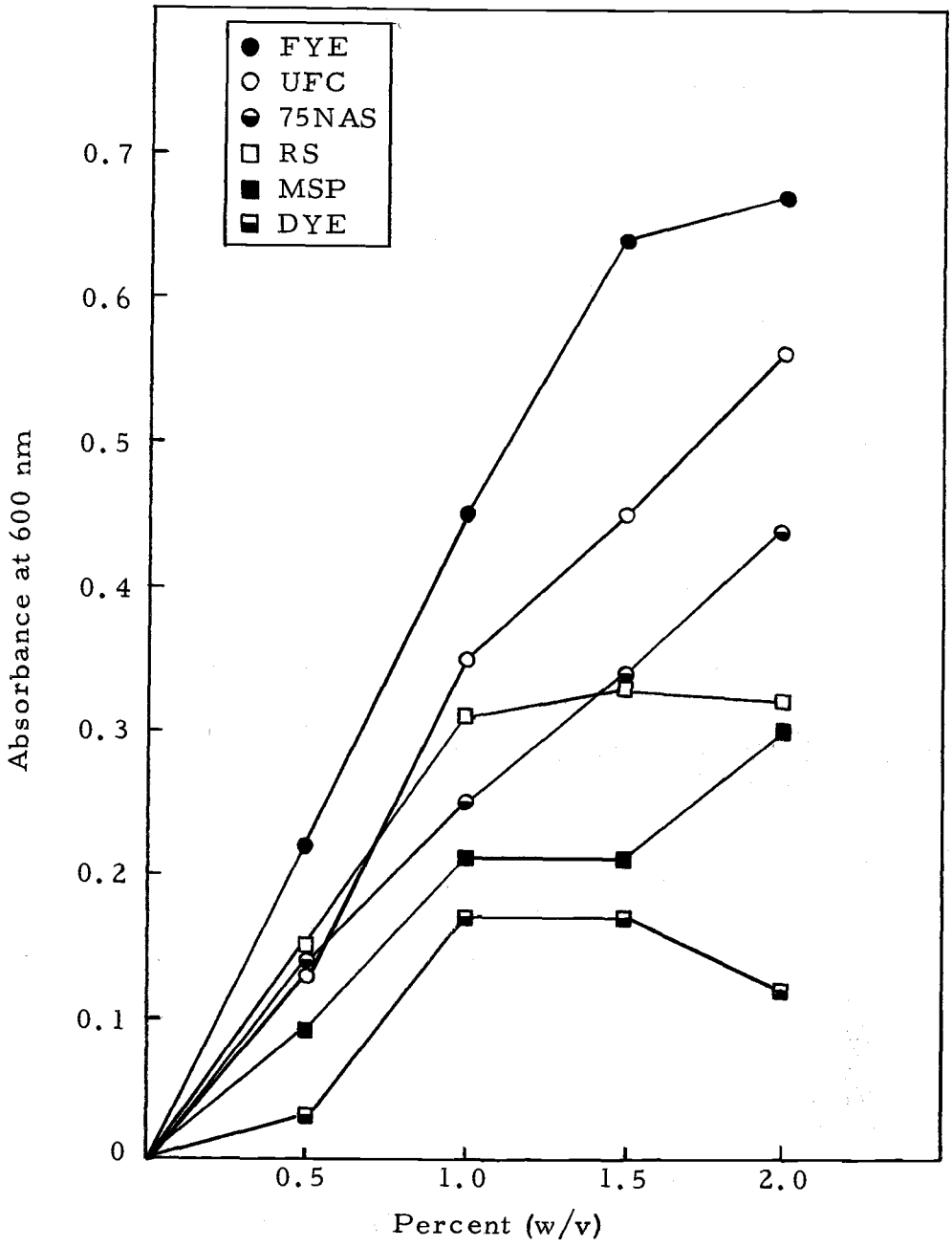

Figure 4. Growth promoting activity of commercial yeast extractives.

To test the importance of the yeast, FYE was prepared from S. carlsbergensis (brewers' yeast), S. cerevisiae (bakers' yeast), and S. exiguus (sourdough yeast). Extractives prepared from S. carlsbergensis were found to be superior to those prepared from the other yeasts, particularly at lower concentrations (Figure 5), suggesting that the uniqueness of FYE involves the method of preparation rather than the yeast from which it is prepared. Moreover, certain complex materials other than yeast extractives are also able to support the growth of sourdough bacteria. The growth promoting activity of two such substances, corn steepwater and fish peptone, are shown in Figure 5. Their diverse natures suggests that stimulatory compounds for sourdough bacteria are widely distributed in nature and are not unique to yeast extractives, which are merely a richer source of such compounds.

Preparation of FYE

The effect of temperature and pH during the preparation of FYE was examined to establish the parameters required for production of yeast extractives with high growth promoting activity for sourdough bacteria.

Results shown in Figure 6 indicate that greater amounts of stimulatory materials were released as the extraction temperature was increased from 60 C to 120 C.

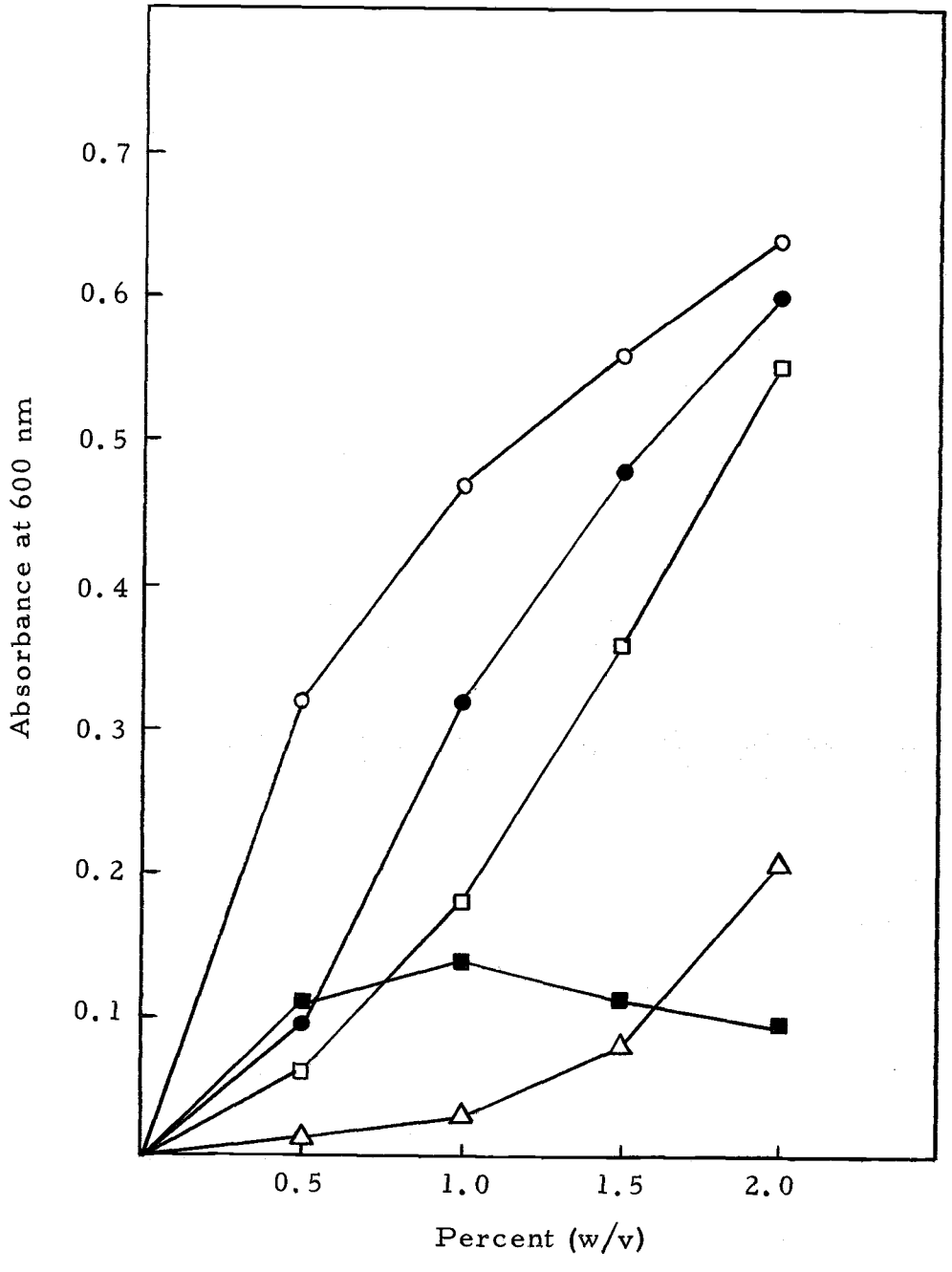

Figure 5. Growth promoting activity of corn steepwater (■ - ■), fish peptone (△ - △), and FYE prepared from S. carlsbergensis (O - O), S. cerevisiae (● - ●), and S. exiguus (□ - □).

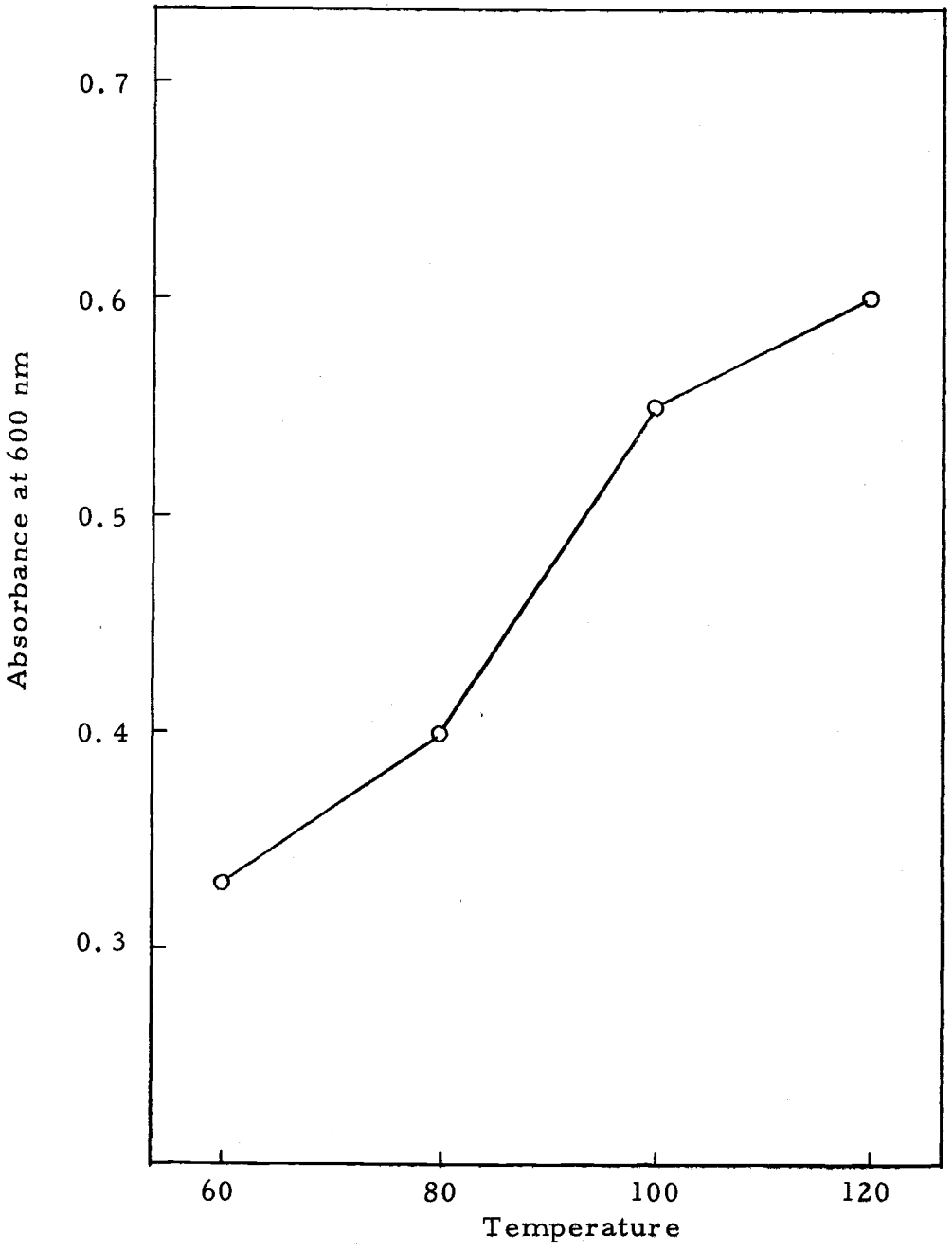

Figure 6. Effect of extraction temperature on the production of FYE.

The natural pH of autoclaved slurries of yeast was generally found to lie between 4.5 and 5.5. A pH of 2.0 during extract preparation caused a slight decrease in activity of the extract, but little effect was noted within a pH range of 3.0 to 10.0 (Figure 7).

Autolysis was found to be a rather poor method for extract production in terms of growth promoting activity for sourdough bacteria, especially at the lower temperatures used (Figure 8). Such results could be due to destruction of stimulatory components during autolysis, or inefficient solubilization of such components. To resolve this matter, the insoluble residue remaining after autolysis was treated as in the preparation of FYE. The resulting extractive contained growth promoting substances which were not released by autolysis (Figure 8).

Fractionation of FYE

Analysis of FYE revealed the composition shown in Table 4. Two fractions were obtained, based on their solubility in an acetone-ethanol mixture: a soluble fraction, FYES, and an insoluble fraction, FYEP. When tested for growth promoting activity in CBM lacking DYE, FYES was found to retain partial activity, while FYEP contained essentially no activity. When the fractions were combined, however, full growth promoting activity was restored (Figure 9).

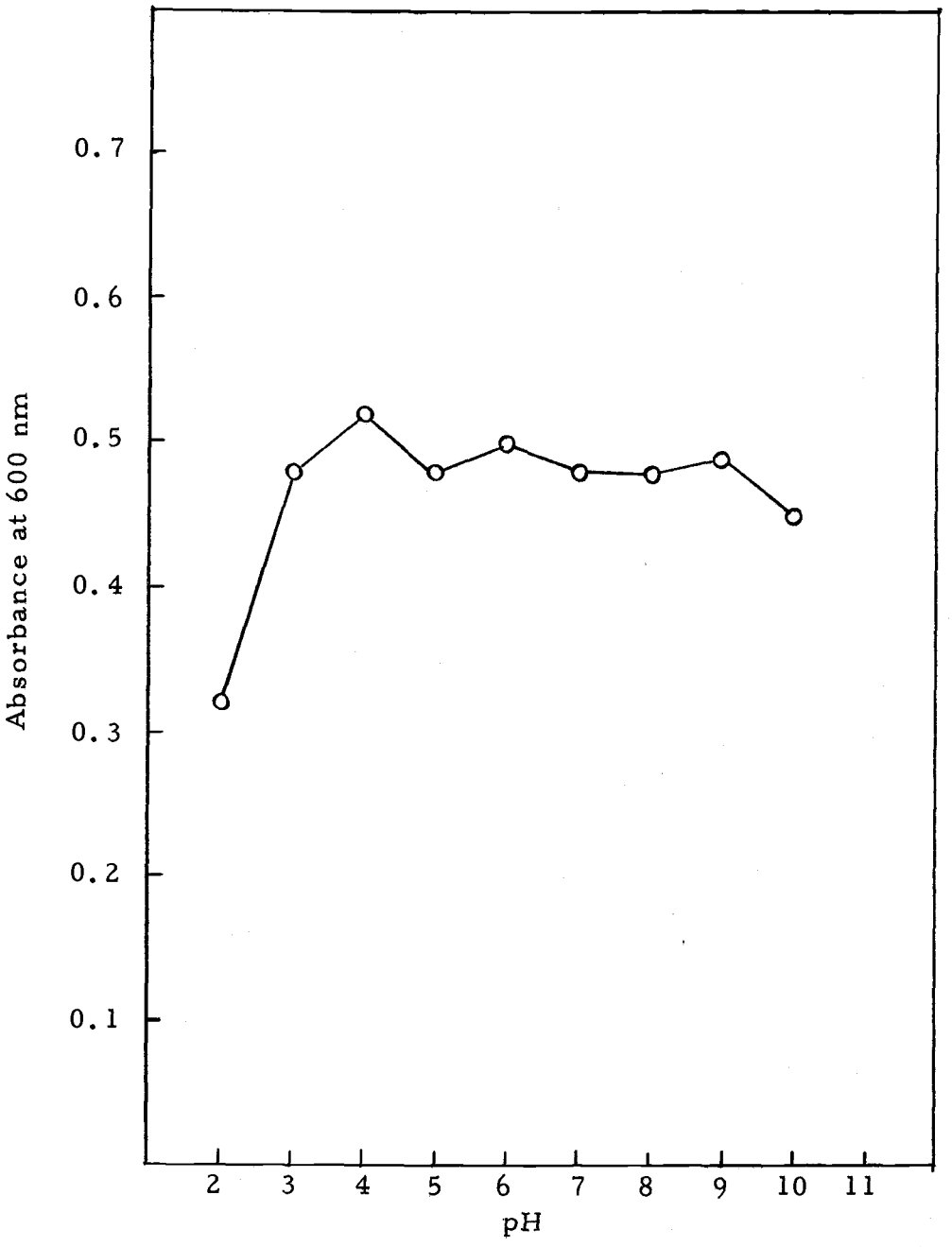

Figure 7. Effect of extraction pH on the production of FYE.

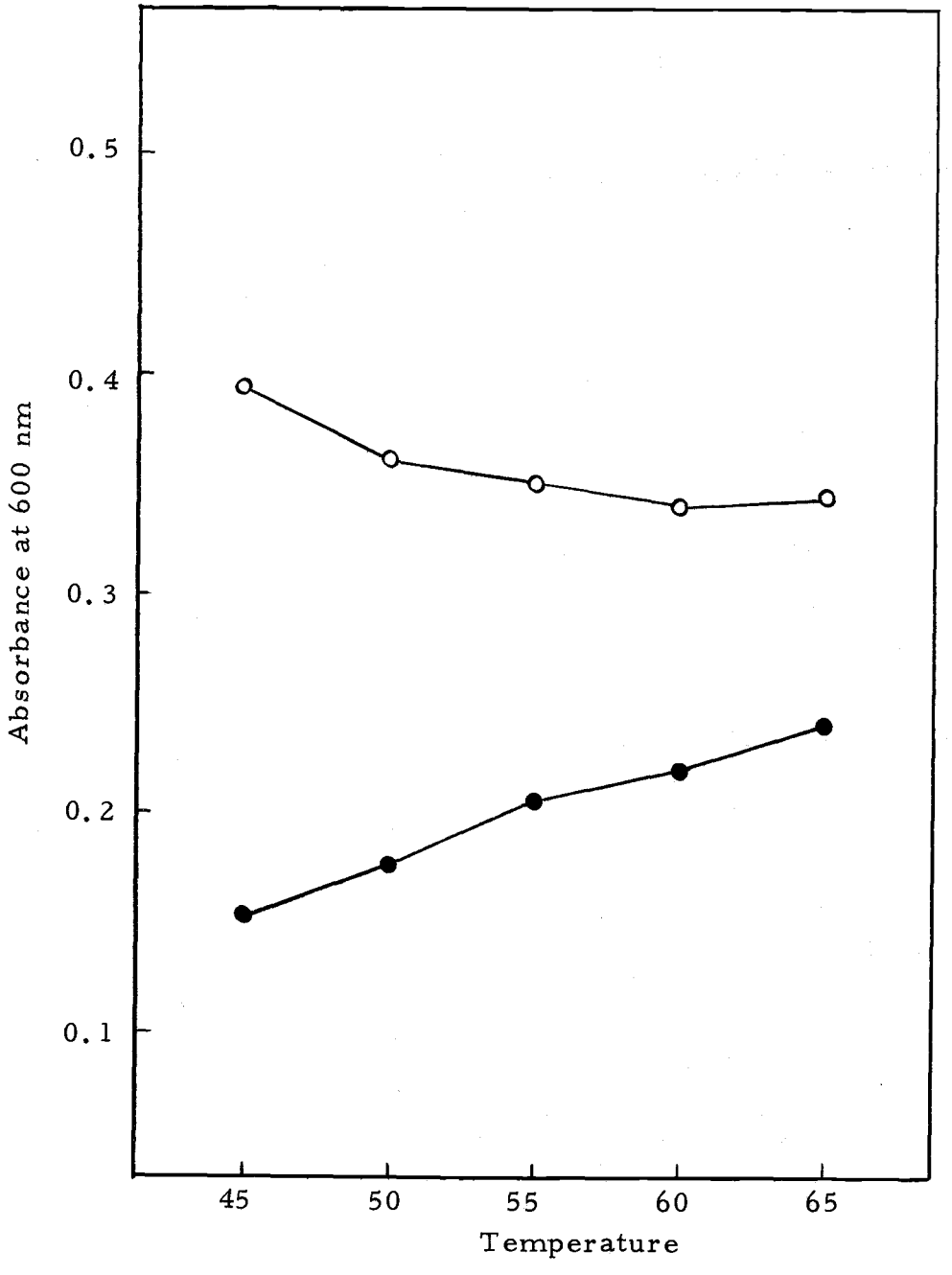

Figure 8. Growth promoting activity of yeast extract produced by autolysis (● - ●) and by hot-water extraction of the residue remaining after autolysis (O - O).

Table 4. Composition of fresh yeast extractives.

Component	mg/g
Protein	153
Carbohydrate	285
RNA	395
DNA	5
Ash	116

95%

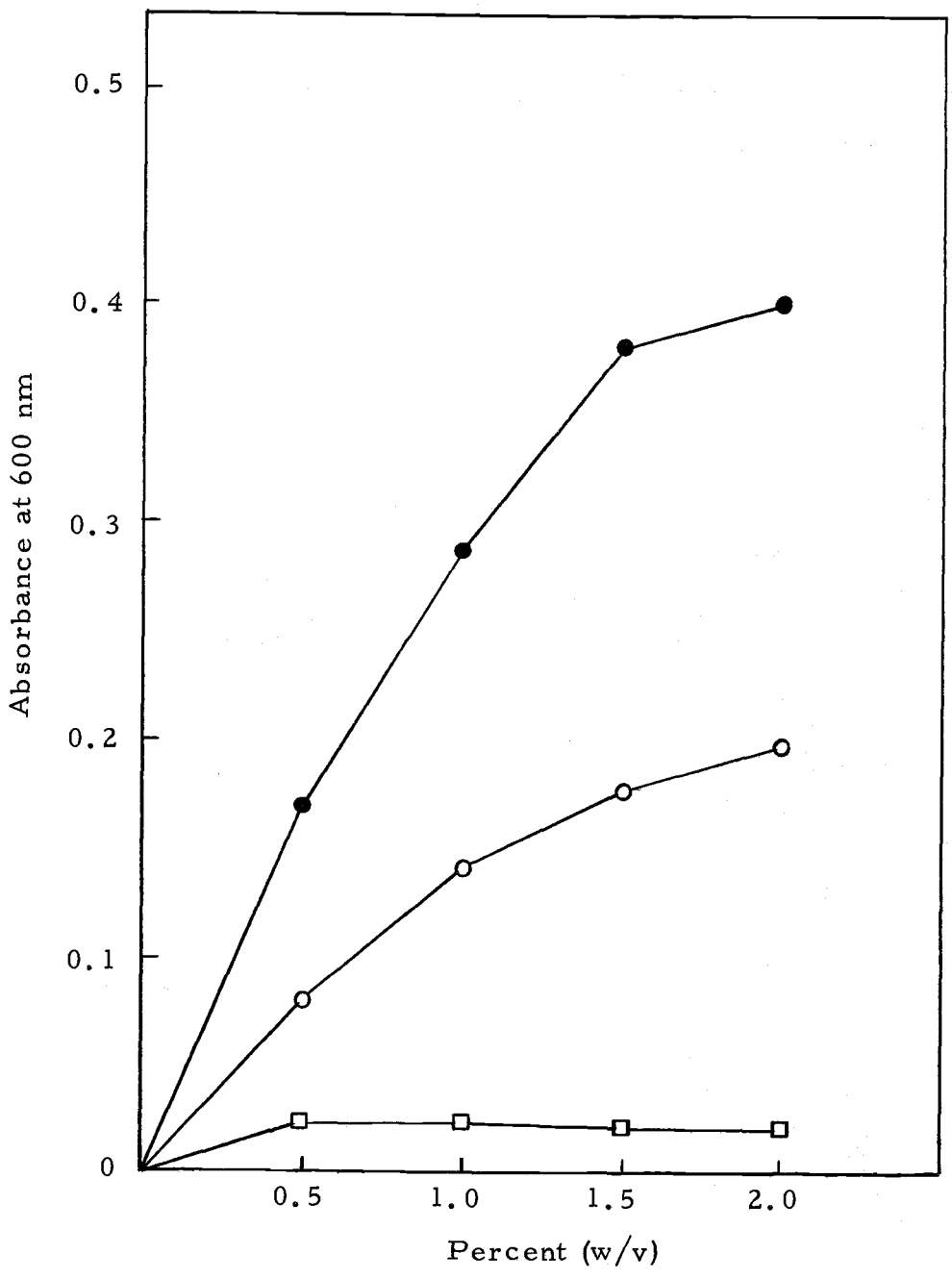

Figure 9. Growth promoting activity of FYE following acetone-ethanol precipitation. (Symbols: O-O, soluble fraction (FYES); □ - □, insoluble fraction (FYEP); ● - ● , FYES + FYEP).

Ashing of the fractions suggested that the active component of FYEP was an inorganic substance, since growth promoting activity in CBM was retained (Figure 10). Addition of divalent cations to CBM at concentrations of 10 μg/ml revealed Mn^{2+} to be highly stimulatory, with a small amount of growth stimulation attributable to Mg^{2+} as well (Figure 11). The Mn^{2+} contents of FYE and some commercial extractives are shown in Table 5. The poorest extractives in terms of growth promoting activity for sourdough bacteria, MSP and DYE, are also the lowest in Mn^{2+} content. However, the activity of FYE cannot be wholly accounted for by its Mn^{2+} content, since other extractives with less activity contain approximately the same amount of this metal.

In order to quantitate the Mn^{2+} requirement of sourdough bacteria, this cation was extracted from SDB and CBM with 8-hydroxyquinoline and added back in known amounts as the sulfate salt. The unextracted media contained Mn^{2+} at concentrations of 0.028 μg/ml (CBM) and 0.14 μg/ml (SDB), while approximately 0.10 μg/ml and 0.20 μg/ml, respectively, were required for maximum growth in these media (Figure 12). It is clear, then, that Mn^{2+} is limiting in CBM, but maximum growth obtained in CBM was considerably less than that observed in SDB, indicating that FYE was supplying, in addition, some factor(s) other than Mn^{2+}. For the isolation of this factor, attention was turned to the FYES fraction of FYE.

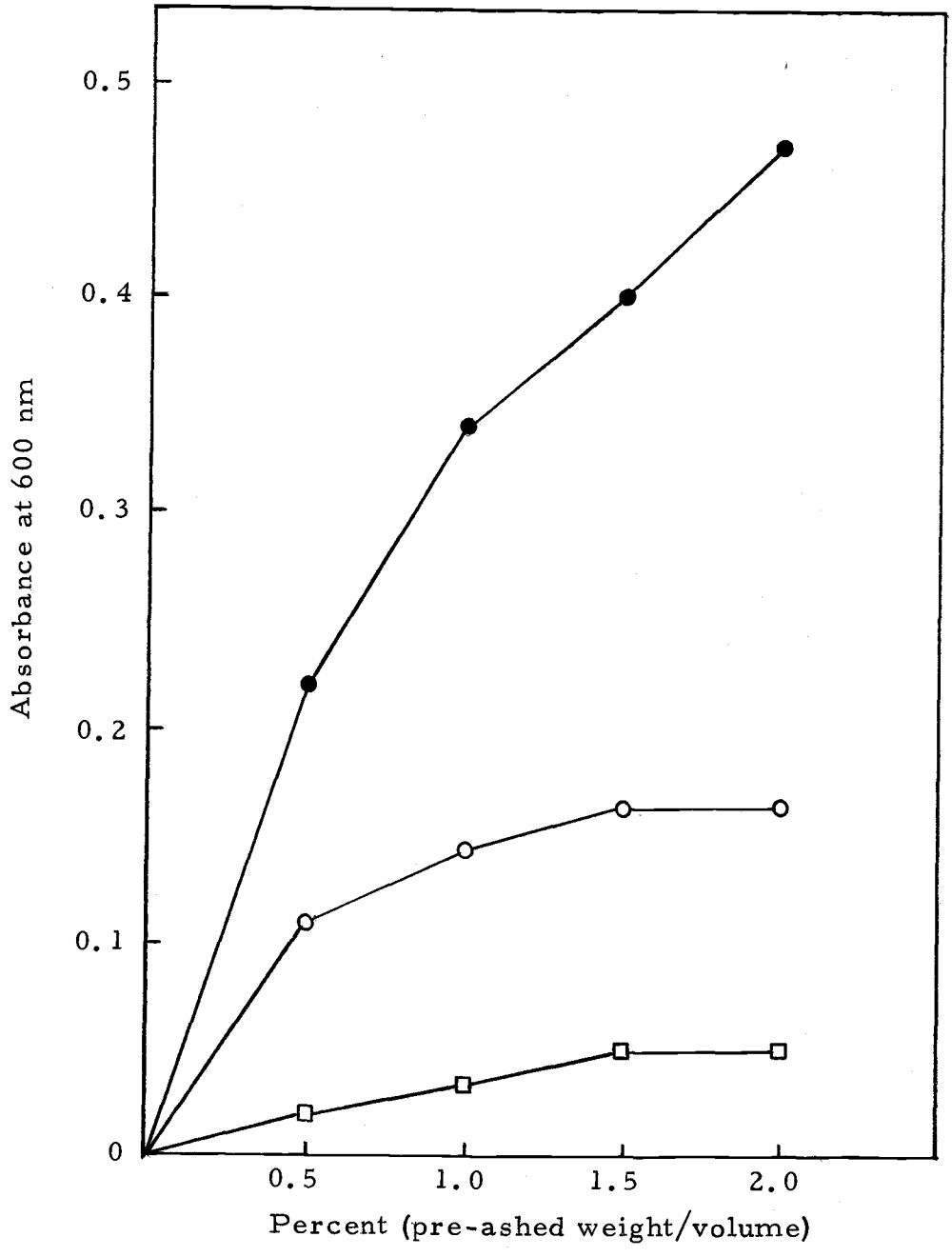

Figure 10. Growth promoting activity of FYE (● - ●) and the ash of FYEP (O - O) and FYES (□ - □).

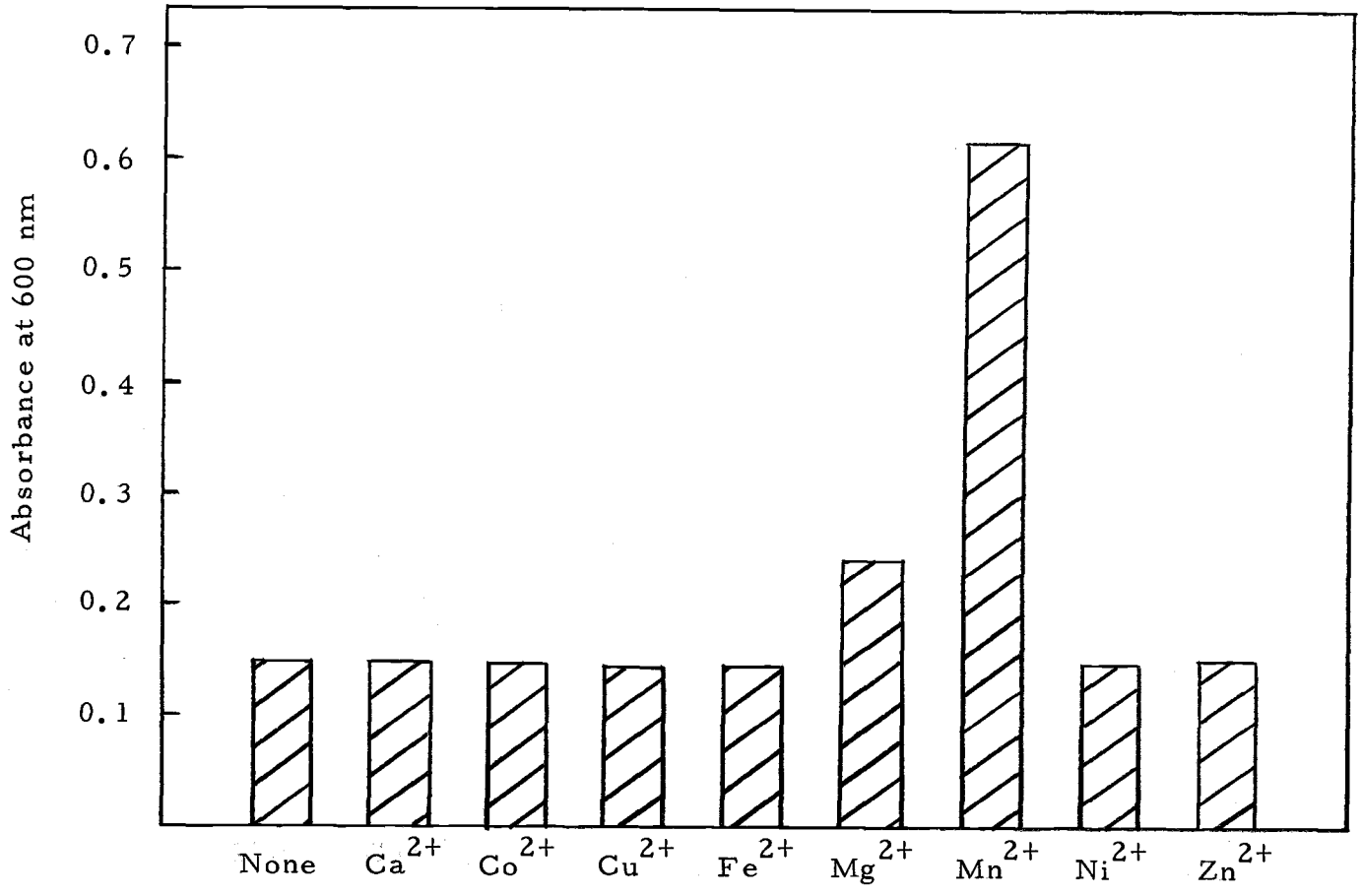

Figure 11. Growth promoting activity of divalent cations added to CBM at concentrations of 10 μg/ml.

Table 5. Manganese content of some substances with stimulatory activity for sourdough bacteria.

Substance	Mn^{2+} Content µg/g
FYE	19
UFC	24
75-NAS	19
RS	22
MSP	<2
DYE	2.5
CS	38

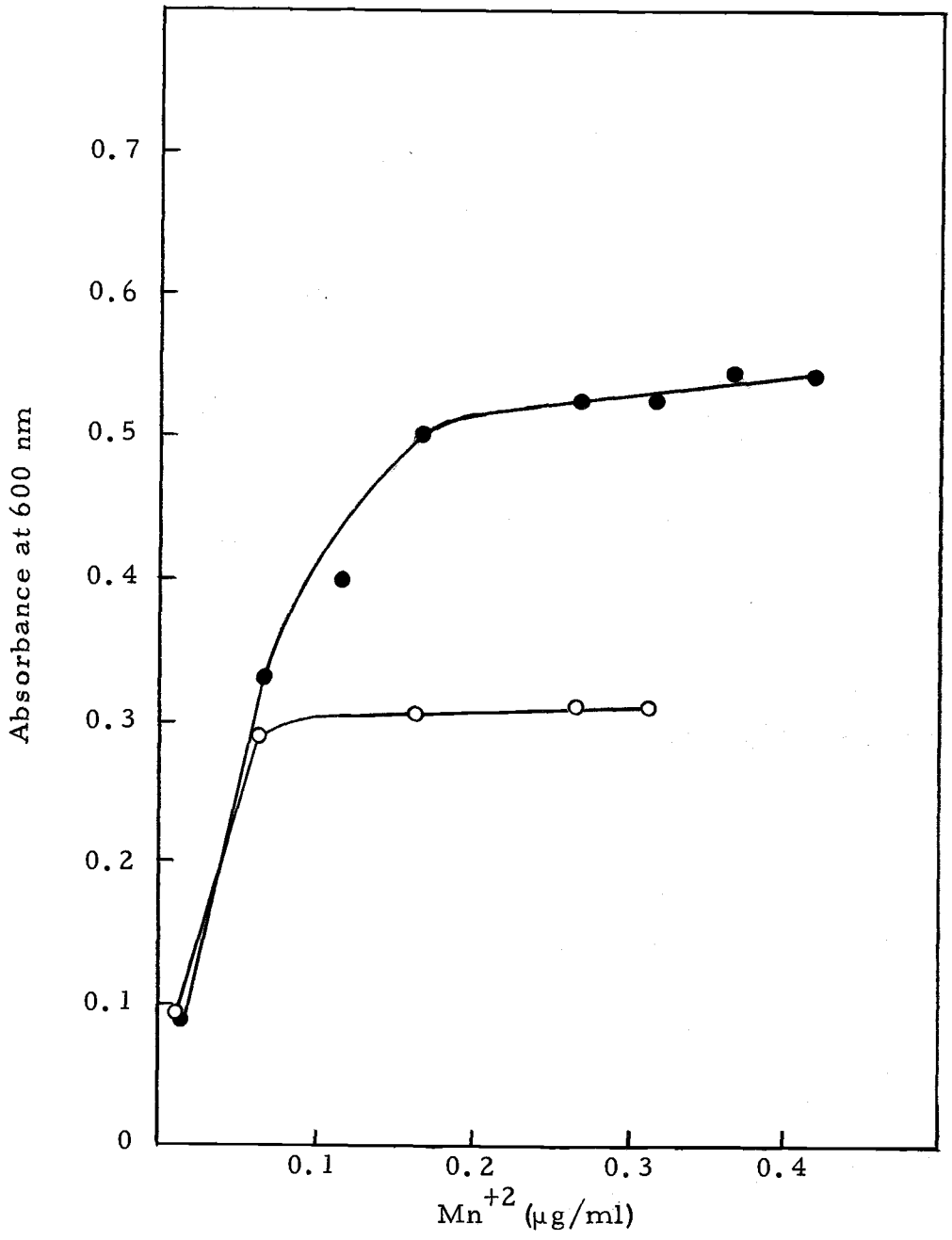

Figure 12. Growth promoting activity of manganous ion added back to extracted SDB (● - ●) and CBM (O - O) media.

Three fractions were obtained on separation of FYES on Bio-Gel P-2, with peak 2 containing the stimulatory material (Figure 13). In addition to material absorbing at 260 nm, this fraction also contained ninhydrin-reactive material and carbohydrate.

The active fraction was applied to a column of DEAE Sephadex and eluted with a gradient of 0.5 to 1.5 M TEAA buffer, pH 4.7, revealing three peaks of ninhydrin-reactive material and one of material absorbing at 260 nm (Figure 14). Growth promoting activity was contained in the first two ninhydrin peaks, which were more fully resolved when the column was eluted with 0.5 M TEAA buffer (Figure 15). The active component was contained in the second ninhydrin peak, but was not resolved from a peak of carbohydrate material.

Ninhydrin-reactive and carbohydrate materials were separated by adsorbing the former on a column of AG50W-X8 cation exchange resin followed by batch elution with 2 N NH_4OH. Growth promoting activity was found to reside with the ninhydrin-reactive fraction (Table 6).

Amino acid analysis of the active fraction revealed the composition shown in Table 7. Eight amino acids were detected in this fraction, with alanine present in greatest quantity. A mixture of amino acids approximating the composition found in the active fraction failed to affect the growth of the sourdough organisms. Hydrolysis of the active fraction revealed the presence of several amino

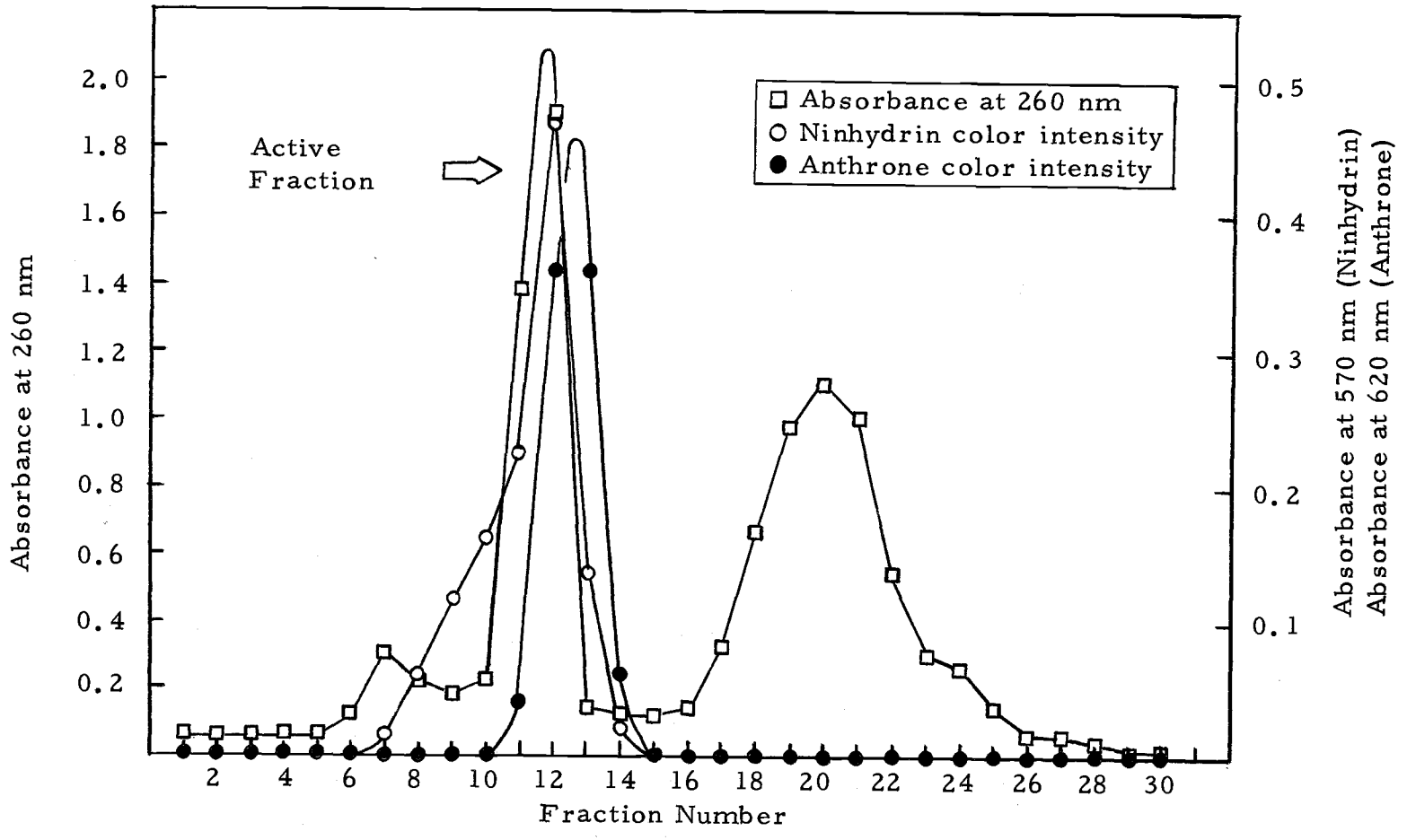

Figure 13. Elution profile of the active fraction from FYE on Bio-Gel P-2.

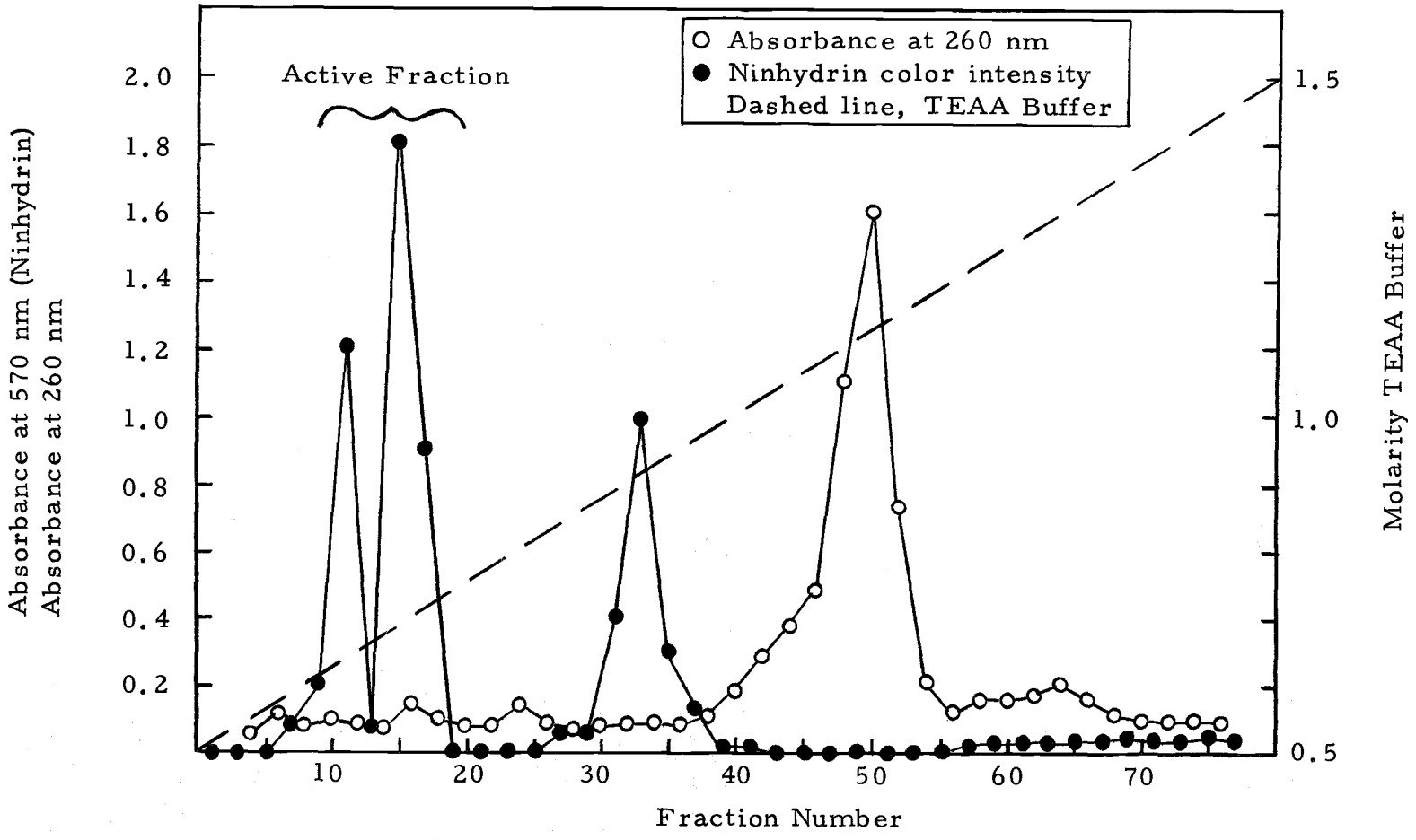

Figure 14. Elution profile of the active fraction from Bio-Gel P-2 on DEAE - Sephadex.

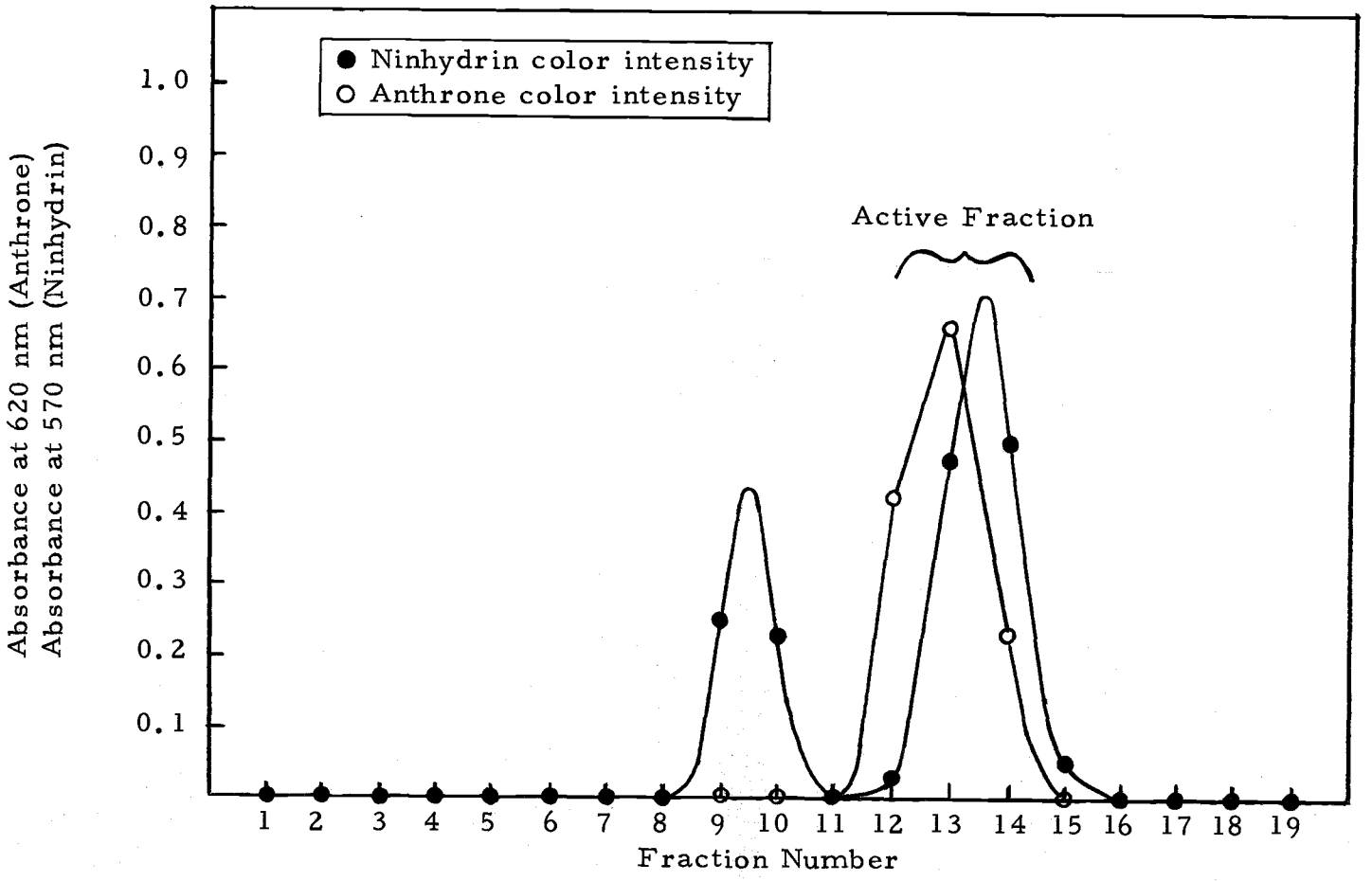

Figure 15. Elution profile of the active fraction from Bio-Gel P-2 on DEAE - Sephadex eluted with 0.5 M TEAA buffer.

Table 6. Fractionation of active fraction from DEAE-Sephadex on AG50W-X8.

Test	Effluent	Eluate
Anthrone	+	-
Ninhydrin	-	+
Growth	-	+

Table 7. Amino acid content of active fraction following chromatography on AG50W-X8.

Amino Acid	Molar Ratio	
	Unhydrolyzed	Hydrolyzed
Alanine	19.5	19.2
Aspartic acid	--	.293
Cystine/2	--	.473
Glutamic acid	--	.654
Glycine	1.16	2.02
Isoleucine	1.05	.945
Leucine	1.25	1.27
Lysine	--	.642
Proline	4.71	4.74
Serine	1.10	1.24
Threonine	1.00	1.00
Valine	2.66	2.63

acids not detected in the unhydrolyzed sample, and an increase in the amount of glycine, suggesting the presence of a peptide.

Free amino acids were destroyed by reaction with ninhydrin, and the active peptide was isolated by chromatography on silica gel G, where it yielded only one ninhydrin positive zone with an Rf of 0.21. The isolated peptide contained aspartic acid, cysteine (expressed as half-cystine since it is oxidized to the latter compound during hydrolysis), glutamic acid, glycine, and lysine. It should be noted that hydrolysis and the rather rigorous treatment during removal of free amino acids may have destroyed certain amino acids present in the original peptide. Assuming that the number of residues in Table 8 is correct and rounding off these values to the nearest whole residue, the approximate molecular weight of the peptide is calculated to be 1,065. This is in agreement with its behavior during gel filtration, at which point it was included by Bio-Gel P-2 (exclusion limit = 1,800).

Equivalent amounts of growth stimulation of sourdough bacteria were typically observed when either the isolated peptide or the original FYE was added to SSBM cultures of these organisms (Table 9). This suggests that the isolated peptide is responsible for the major portion of the stimulation of the test organisms by FYE in this medium.

Table 8. Amino acid composition of peptide isolated from FYE.

Amino Acid	No. Residues[1]
Aspartic acid	1.0
Cystine/2	1.4
Glutamic acid	2.2
Glycine	2.9
Lysine	2.2

[1] Calculated by dividing the micromolar values by the micromolar value of the amino acid present in least abundance.

Table 9. Stimulation of growth of sourdough bacteria by FYE and by the peptide isolated from FYE.

Substrate	A_{600}
SSBM	0.03
SSBM + 0.5% FYE	0.41
SSBM + peptide[a]	0.36

[a]Derived from FYE equivalent to 1% in test medium to allow for losses during fractionation.

Vitamin Requirements of Sourdough Bacteria

The vitamin and purine-pyrimidine requirements of the sourdough bacteria are shown in Table 10. All four strains required thiamine, nicotinic acid, and calcium pantothenate, while strain C required folic acid as well. Strain differences became more apparent in the response of the organisms to purines and pyrimidines. Strain B required adenine, while strains C and L required uracil. In addition, guanine was stimulatory for strain C. The purine-pyrimidine requirement of strain T could be partially fulfilled by adenine, guanine, or uracil, but all three were needed for maximal growth.

Table 10. Vitamin and purine-pyrimidine requirements of sourdough bacteria.

Compound	Strain			
	B	C	L	T
Thiamine	+	+	+	+
Riboflavin	-	-	-	-
Nicotinic acid	+	+	+	+
Calcium pantothenate	+	+	+	+
Pyridoxamine	-	-	-	-
Para-aminobenzoic acid	-	-	-	-
Biotin	-	-	-	-
Folic acid	-	+	-	-
Adenine sulfate	+	-	-	+[1]
Guanine HCl	-	S	-	+[1]
Uracil	-	+	+	+[1]
Xanthine	-	-	-	-

Key: + = required, - = not required, S = stimulatory

[1] Any one of these compounds supports partial growth; all needed for maximal growth.

DISCUSSION

The generation time of sourdough bacteria in SD broth is about half that observed during sourdough fermentation. It is apparent, then, that SD broth presents a less rigorous environment for the bacteria than their natural habitat. This is probably the result of higher concentrations of required nutrients, the presence of nutrients in a more readily available form, less restricted diffusion of nutrients and waste products in the less viscous medium, or a combination of these factors.

The exceptional growth promoting properties of FYE appear to result from its method of preparation. Commercial yeast extractives, due to considerations of expediency, efficiency, and economy, are generally prepared from the water soluble portion of autolyzed yeast (Acraman, 1966). Results of this study, however, indicate that autolysis is not an efficient method of extracting growth promoting substances important in the nutrition of sourdough bacteria (Figure 8). Rather, extraction efficiency was found to depend upon the temperature during hot-water extraction of whole yeast cells. While temperatures above 121 C were not tested, extraction efficiency increased with increasing temperatures up to this limit, within a pH range of 3.0 to 10.0 (Figures 6 and 7).

Manganese was found to be required at concentrations of approximately 0.2 μg/ml for maximal growth of the sourdough organisms, a requirement which could be met by the incorporation of FYE into basal media. A similar requirement was demonstrated for various lactic acid organisms by Bentley, Snell, and Phillips (1947), who reported a linear growth response over the range of 0.1 to 0.3 μg of manganese per ml of medium. Growth stimulation of many lactic acid bacteria by this element has been reported through the years. Zlataroff and Kaltschewa (1936) reported that S. lactis produced greater acidities in milk cultures when manganese salts were added, and Möller (1939) demonstrated the requirement of L. plantarum for this element. Evans and Niven (1951) noted that the addition of manganese to a complex medium promoted intensive growth of heterofermentative lactobacilli involved in the greening of cured meat products, and Stamer, Albury, and Pederson (1964) demonstrated a manganese requirement in 63 of 71 strains of lactic acid bacteria of the genera Lactobacillus, Leuconostoc, and Pediococcus.

The manganese content of yeast extractives is probably dependent upon the growth conditions during yeast production. Yeast cells bind divalent cations to the cell surface, but cations so bound are completely exchangeable with the cations of the medium in the absence of glucose (Rothstein and Hayes, 1956). Certain of these cations, particularly Mg^{2+} and Mn^{2+}, can be incorporated into a

non-exchangeable pool in the yeast cell by a specific transport system, provided that glucose is available and that phosphate is also absorbed (Jennings, Hooper, and Rothstein, 1958; Fuhrmann and Rothstein, 1968). Yeasts harvested from depleted media as byproducts of fermentation may therefore be expected to contain lower concentrations of manganese than organisms harvested during vigorous fermentation.

The roles of manganese in cellular processes are diverse, including prevention of cellular autolysis (MacLeod, 1951), contributing to the stability and function of ribosomes (Sheard, et al., 1967), and activation of nucleic acid polymerase (Pogo, et al., 1967), cell wall synthesizing enzymes (Anderson, et al., 1966), and a host of other enzymatic reactions (Lehninger, 1950). Magnesium can replace manganese requirements for many functions, but not in sporulation or secondary metabolite biosynthesis (Weinberg, 1970), or, apparently, in the nutrition of sourdough bacteria. In contrast to the relatively non-specific divalent cation accumulating mechanism of yeast, a highly specific active transport system for manganese in Escherichia coli has been described (Silver, Johnseine, and King, 1970; Bhattacharyya, 1970). The actual site of incorporation of manganese into sourdough bacterial cells and its diversified modes of action, however, remain to be elucidated.

The mineral composition of wheat and flour is variable and is affected by heredity and by environmental factors including soil type, fertilization, and weather (Greaves, Bracken, and Hirst, 1940; El Gindy, Lamb, and Burrell, 1957). Analysis of 40 flours representing five varieties of wheat harvested at eight locations, however, showed an average manganese content of 0.059 mg/g (Bequette, et al., 1963). This suggests that this element is not limiting during sourdough fermentations, since pure cultures of sourdough bacteria require only 0.2 μg/ml for optimal growth in SD broth (Figure 12).

Since the discovery of strepogenin by Woolley (1941), considerable research has been directed toward the importance in bacterial nutrition of peptides isolated from a variety of sources (Payne and Gilvarg, 1971). In view of the marked structural specificity required in vitamins and other biologically active substances, the role of peptides in bacterial nutrition is somewhat of an anomaly. The inability to pin down a single, pure strepogenin factor led Woolley and Merrifield (1958, 1963) to conclude that it is not possible to adequately relate peptide structure or composition with strepogenin activity. They demonstrated that no single amino acid residue was essential, for each could generally be exchanged for related ones without complete loss of biological activity. On the other hand, many peptides were inactive, and in some cases very small changes in structure completely abolished activity. When serine was replaced

by threonine in serylhistidylleucylvalylglutamic acid, for example, the resultant peptide was totally without biological activity, even though this peptide differed from the active one only by having one H atom replaced by $-CH_3$ (Merrifield, 1958). No active peptide was found which did not contain either serine or cysteine (Merrifield and Woolley, 1958), but the mere presence of serine or cysteine was not enough, for many peptides which contained one or the other of these were inactive (Woolley, et al., 1955). Moreover, each residue could generally be replaced by something else, but not just by anything else, presenting a very confusing picture indeed. Similarly, the amino acid sequence was relatively unimportant, for the order could be varied with retention of activity. The size of the peptide was important, however, because it could be either too large or too small, with an average of seven residues being optimal. Thus, no peptide smaller than five amino acid residues possessed significant activity, nor did very large ones such as intact proteins. The addition of an arginine residue to the carboxyl end of leucylvalylcysteinylglycylglutamic acid, for example, resulted in a 20-fold increase in activity (Tritsch and Woolley, 1960).

Specific amino acid transport systems in bacteria have been extensively studied (Payne and Gilvarg, 1971) and are metabolically justifiable for the uptake of the relatively small number of biologically significant amino acids. In the case of peptides, however, the

extremely large number of structural combinations and permutations, 400 for dipeptides, 8,000 for tripeptides, and so on, precludes the existence of a transport system specific for each one. On the other hand, the requirement for a source of energy for peptide uptake has been demonstrated (Smith, Archer, and Dunn, 1970) and inhibition of energy coupling has been shown to prevent peptide uptake (Mayshak, et al., 1966). Present evidence suggests that only one oligopeptide transport system exists in E. coli and that it is able to handle oligopeptides formed from any of the natural amino acids. However, certain structural specifications must be met for transport to occur in this organism. A free N-terminal α-amino group is apparently essential for peptide uptake: acetylated arginine and lysine oligopeptides were not nutritionally active for whole cells, but were cleaved by peptidases from toluene-treated cells, suggesting that the nutritional ineffectiveness of the peptides must derive from their inability to enter the cell (Losick and Gilvarg, 1966). Conversely, uptake is not significantly affected by loss of the C-terminal carboxyl group. Lysylcadaverine peptides (cadaverine is the diamine obtained by decarboxylation of lysine) were able to enter and support growth of E. coli (Payne and Gilvarg, 1968a).

Chain length of a peptide is an important determinant of its ability to enter the cell; in E. coli exclusion begins with five to six residues. Higher excluded peptides are unable to competitively

inhibit the uptake of lower peptides, suggesting that the discrimination against larger peptides is unlikely to be exerted at the transport system itself, which is shared by all peptides. Payne and Gilvarg (1968b) speculated that the cell wall may act as a molecular sieve preventing molecules above a certain size from reaching the peptide transport system. It should be noted, however, that the peptide uptake mechanism elucidated for E. coli may not be a general phenomenon. Studies by Pittman, Lakshmanan, and Bryant (1967) with Bacteroides ruminicola, for example, seem to suggest that both large and small peptides can enter this organism with equal facility.

The explanation of peptide activity offered by Kihara and Snell (1960a, b) as presented in the literature review, i. e., that peptides function by supplying amino acids in a readily available form, is the one which is generally accepted today. To date, no unique strepogenin peptide has been isolated, but an apparent requirement for peptides of relatively specific structure can result where impediments exist simultaneously to utilization of several different amino acids. This may be the case with the peptide active for sourdough bacteria since classical sources of strepogenin activity, such as partial casein hydrolysates, do not exhibit significant growth promoting activity for these organisms.

Jones and Carnegie (1969) described the isolation and characterization of disulfide peptides from wheat flour in which the average

length of peptide chain was of the order of 9 or 10 residues. Such peptides are similar to that isolated in this study, suggesting that perhaps the sourdough bacteria have developed a preference for accumulating peptides in an environment rich in such compounds. In this vein, a highly restrictive system in Bacteroides ruminicola has been described in which the organism is unable to accumulate free amino acids, but can readily take in peptides (Pittman and Bryant, 1964).

However, one should not dismiss the possibility that small peptides may produce more direct biological effects. In this regard, it is interesting to note that a tripeptide of glutamic acid, histidine, and proline functions as a thyrotropin releasing hormone in man and other species (Bowers, et al., 1970; Burgus, et al., 1970), and several small growth controlling peptides for mammalian cells have been described (Tritsch and Grahl-Nielsen, 1969).

Whether the observed growth stimulation by peptides reflects an inherent need for such compounds by the sourdough organisms or is merely an artifact of a nutritionally unbalanced medium must await further study for a definitive answer. Moreover, not only do such factors have possible applications in industrial fermentations, but their role in bacterial, and possibly in human nutrition as well, should be further elucidated.

SUMMARY

San Francisco sourdough bacteria (Lactobacillus sanfrancisco) are responsible for the unique flavor and resultant popularity of San Francisco sourdough French bread. Because of their fastidious nature and unknown nutritional requirements, a great deal of difficulty was encountered during attempts at initial isolation of the organisms in pure culture from sourdough fermentations. For successful isolation, a requirement for a laboratory-prepared hot-water extract of yeast (FYE) was demonstrated which could not be fully satisfied by a variety of complex infusions, partial protein hydrolysates, or commercial yeast extractives. The research which culminated in this thesis was directed toward this requirement in an effort to determine why FYE is better able to support rapid growth of the sourdough organisms than apparently similar complex materials, and which components are required or stimulatory in their nutrition.

The exceptional growth promoting properties of FYE were found to result from its method of preparation: most commercial extractives are produced by autolysis, a process which was shown to be less efficient in releasing growth promoting materials for sourdough bacteria than the hot-water extraction procedure used for the preparation of FYE. Materials supplied by FYE were found to include

manganous ion, several vitamins and nucleic acid bases, and a short peptide, with the latter compound accounting for the unique growth promoting activity of FYE.

Sourdough bacteria were shown to require a manganese concentration of approximately 0.2 μg/ml for maximal growth, a level which could be met in basal media by the incorporation of FYE, and some, but not all, of the commercial extractives tested. In addition, an organic factor was required which was isolated and identified as a peptide containing aspartic acid, cysteine, glutamic acid, glycine, and lysine, having a chain length of about nine residues and a molecular weight of approximately 1,065.

The sourdough organisms were all found to require thiamine, nicotinic acid, and calcium pantothenate for growth in the medium used, while strain C, in addition, requires folic acid. One or more of the nucleic acid bases adenine, guanine, and uracil were demonstrated to be required or stimulatory for all strains.

BIBLIOGRAPHY

Acraman, A. R. 1966. Processing brewers yeasts. Process Biochemistry 1:313-317.

Alberts, A. W., P. W. Majerus and P. R. Vagelos. 1965. Acyl carrier protein. VI. Purification and properties of β-ketoacyl acyl carrier protein synthetase. Biochemistry 4:2265-2274.

Anderson, J. S. et al. 1966. Biosynthesis of the peptidoglycan of bacterial cell walls. I. Utilization of uridine diphosphate acetylmuramyl pentapeptide and uridine diphosphate acetylglucosamine for peptidoglycan synthesis by particulate enzymes from Staphylococcus aureus and Micrococcus lysodeikticus. Archives of Biochemistry and Biophysics 116:487-515.

Angier, R. B. et al. 1946. The structure and synthesis of the liver L. casei factor. Science 103:667-669.

Ayers, S. H. and P. Rupp. 1920. Extracts of pure dry yeast for culture media. Journal of Bacteriology 5:89-98.

Bentley, O. G., E. E. Snell and P. H. Phillips. 1947. A microbiological method for the determination of manganese. The Journal of Biological Chemistry 170:343-350.

Bequette, R. K. et al. 1963. Mineral composition of gluten, starch and water-soluble fractions of wheat flour and its relationship to flour quality. Agronomy Journal 55:537-542.

Bhattacharyya, P. 1970. Active transport of manganese in isolated membranes of Escherichia coli. Journal of Bacteriology 104: 1307-1311.

Blakley, R. L. 1965. Cobamides and ribonucleotide reduction. I. Cobamide stimulation of ribonucleotide reduction in extracts of Lactobacillus leichmanii. The Journal of Biological Chemistry 240:2173-2180.

Bowers, C. Y. et al. 1970. Activity and specificity of synthetic thyrotropin-releasing hormone in man. Biochemical and Biophysical Research Communications 39:352-355.

Burgus, R. et al. 1970. Characterization of ovine hypothalamic hypophysiotropic TSH-releasing factor. Nature 226:321-325.

Caldwell, I. C. 1969. Ion-exchange chromatography of tissue nucleotides. Journal of Chromatography 44:331-341.

Ceriotti, G. 1952. A microchemical determination of deoxyribonucleic acid. The Journal of Biological Chemistry 198:297-303.

Cheldelin, V. H., E. H. Hoag and H. P. Sarett. 1945. The pantothenic acid requirements of lactic acid bacteria. Journal of Bacteriology 49:41-45.

Clark, J. M. 1964. Experimental biochemistry. San Francisco, W. H. Freeman and Company. 228 p.

Cogan, T. M., S. E. Gilliland and M. L. Speck. 1968. Identification of stimulants for Lactobacillus bulgaricus in tomato juice. Applied Microbiology 16:1215-1219.

Cornfield, A. H. and A. G. Pollard. 1950. The use of tetramethyldiamino diphenylmethane for the determination of small amounts of manganese in plant material and soil extracts. Journal of the Science of Food and Agriculture 1:107-109.

Craig, J. A. and E. E. Snell. 1951. The comparative activities of pantethine, pantothenic acid, and coenzyme for various microorganisms. Journal of Bacteriology 61:283-291.

Deibel, R. H., J. B. Evans and C. F. Niven, Jr. 1957. Microbiological assay for thiamin using Lactobacillus viridescens. Journal of Bacteriology 74:818-821.

Dische, Z. and E. Borenfreund. 1957. A new color reaction for the determination of aldopentose in presence of other saccharides. Biochimica et Biophysica Acta 23:639-642.

Dubnoff, J. W. and E. Bartron. 1956. The activation of protein sulfhydryl groups by vitamin B_{12}. Archives of Biochemistry and Biophysics 62:86-90.

Eastcott, E. V. 1928. Wildiers' bios. The isolation and identification of "Bios I." The Journal of Physical Chemistry 32:1094-1111.

El Gindy, M. M., C. A. Lamb and R. C. Burrell. 1957. Influence of variety, fertilizer treatment, and soil on the protein content and mineral composition of wheat, flour, and flour fractions. Cereal Chemistry 34:185-195.

Evans, J. B. and C. F. Niven, Jr. 1951. Nutrition of the heterofermentative lactobacilli that cause greening of cured meat products. Journal of Bacteriology 62:599-603.

Eyssen, H. et al. 1965. Nucleotide requirements of Lactobacillus acidophilus variants isolated from the crops of chicks. Antonie van Leeuwenhoek 31:241-248.

Fox, E. N. 1961. Peptide requirements for the synthesis of streptococcal proteins. The Journal of Biological Chemistry 236:166-171.

Fuhrmann, G. and A. Rothstein. 1968. The transport of Zn^{2+}, Co^{2+} and Ni^{2+} into yeast cells. Biochimica et Biophysica Acta 163:325-330.

Gentry, C. H. R. and L. G. Sherrington. 1950. Extraction and photometric estimation of some metals with 8-hydroxyquinoline. Analyst 75:17-21.

Gornall, A. G., C. J. Bardawill and M. M. David. 1948. Determination of serum proteins by means of the biuret reaction. The Journal of Biological Chemistry 177:751-766.

Grant, C. L. and D. Pramer. 1962. Minor element composition of yeast extract. Journal of Bacteriology 84:869-870.

Greaves, J. E., A. F. Bracken and C. T. Hirst. 1940. The influence of variety, season and green manures upon the composition of wheats. Journal of Nutrition 19:179-186.

Greene, R. D., A. J. Brook and R. B. McCormack. 1949. Some conditions which affect the assay of vitamin B_{12} with Lactobacillus lactis Dorner. The Journal of Biological Chemistry 178:999-1000.

Gyllenberg, H., M. Rossander and P. Roine. 1953. A strain of Lactobacillus bifidus which requires strepogenin. The Journal of General Microbiology 9:190-198.

György, P. and C. S. Rose. 1955. Further observations on the metabolic requirements of Lactobacillus bifidus var. pennsylvanicus. Journal of Bacteriology 69:483-490.

Henderson, L. M. and E. E. Snell. 1948. A uniform medium for determination of amino acids with various microorganisms. The Journal of Biological Chemistry 172:15-29.

Hendlin, D., L. K. Koditschek and M. H. Soars. 1953. Investigations on the biosynthesis of citrovorum factor by lactic acid bacteria. Journal of Bacteriology 65:466-471.

Hitchings, G. H. et al. 1950. Antagonists of nucleic acid derivatives. I. The Lactobacillus casei model. The Journal of Biological Chemistry 183:1-9.

Hough, J. S. and J. S. Maddox. 1970. Yeast autolysis. Process Biochemistry 5:50-52.

Hutner, S. H. 1972. Inorganic nutrition. In: Annual review of microbiology, ed. by C. E. Clifton, S. Raffel and M. P. Starr. Vol. 26, Palo Alto, Annual Reviews Inc. p. 313-346.

Ikawa, M. and J. S. O'Barr. 1956. The nature of some growth stimulatory substances for Lactobacillus delbrueckii. Journal of Bacteriology 71:401-405.

Jaenicke, L. 1964. Vitamin and coenzyme function: vitamin B_{12} and folic acid. In: Annual review of biochemistry, ed. by E. E. Snell et al. Vol. 33, Palo Alto, Annual Reviews Inc. p. 287-312.

Jennings, D. H., D. C. Hooper and A. Rothstein. 1958. The participation of phosphate in the formation of a "carrier" for the transport of Mg^{++} and Mn^{++} ions into yeast cells. Journal of General Physiology 41:1019-1026.

Jones, I. K. and P. R. Carnegie. 1969. Isolation and characterisation of disulphide peptides from wheat flour. Journal of the Science of Food and Agriculture 20:54-60.

Jones, D. and P. H. A. Sneath. 1970. Genetic transfer and bacterial taxonomy. Bacteriological Reviews 34:40-81.

Joslyn, M. A. 1955. Yeast autolysis. I. Chemical and cytological changes involved in autolysis. Wallerstein Laboratories Communications 18:107-122.

Joslyn, M. A. and D. C. Vosti. 1955. Yeast autolysis. II. Factors influencing the rate and extent of autolysis. Wallerstein Laboratories Communications 18:191-205.

Kihara, H. and E. E. Snell. 1960a. Peptides and bacterial growth. VIII. The nature of strepogenin. The Journal of Biological Chemistry 235:1409-1414.

_____ 1960b. Peptides and bacterial growth. IX. Release of double inhibitions with single peptides. The Journal of Biological Chemistry 235:1415-1418.

Kitay, E., W. S. McNutt and E. E. Snell. 1950. Deoxyribosides and vitamin B_{12} as growth factors for lactic acid bacteria. Journal of Bacteriology 59:727-738.

Kitay, E. and E. E. Snell. 1950. Some additional nutritional requirements of certain lactic acid bacteria. Journal of Bacteriology 60:49-56.

Kline, L. and T. F. Sugihara. 1971. Microorganisms of the San Francisco sour dough bread process. II. Isolation and characterization of undescribed bacterial species responsible for the souring activity. Applied Microbiology 21:459-465.

Kline, L., T. F. Sugihara and L. B. McCready. 1970. Nature of the San Francisco sour dough French bread process. I. Mechanics of the process. The Bakers Digest 44:48-50.

Knight, B. C. J. G. 1937a. The nutrition of Staphylococcus aureus: nicotinic acid and vitamin B_1. The Biochemical Journal 31: 731-737.

_____ 1937b. The nutrition of Staphylococcus aureus. The activities of nicotinamide, aneurin (vitamin B_1) and related compounds. The Biochemical Journal 31:966-973.

Koburger, J. A., M. L. Speck and L. W. Aurand. 1963. Identification of growth stimulants for Streptococcus lactis. Journal of Bacteriology 85:1051-1055.

Kögl, F. and B. Tönnis. 1936. Über das bios-problem. Darstellung von Krystallisierten Biotin aus Eigelb. 20. Mitteilung über pflanzliche Wachstumsstoffe. Hoppe-Seyler's Zeitschrift für Physiologische Chemie 242:43-73.

Koser, S. A. 1968. Vitamin requirements of bacteria and yeasts. Springfield, Illinois, Charles C. Thomas. 663 p.

Koser, S. A. and B. J. Fischer. 1950. Vitamin requirements of oral lactobacilli. Journal of Dental Research 29:760-773.

Koser, S. A. and J. L. Thomas. 1953. Activity of some vitamin derivatives and related compounds for oral lactobacilli. The Journal of Infectious Diseases 93:192-199.

Lampen, J. O. and T. P. Wang. 1952. The mechanism of action of Lactobacillus pentosus nucleosidase. The Journal of Biological Chemistry 198:385-395.

Lardy, H. A., R. L. Potter and R. H. Burris. 1949. Metabolic functions of biotin. I. The role of biotin in bicarbonate utilization by Lactobacillus arabinosus studied with C^{14}. The Journal of Biological Chemistry 179:721-731.

Lehninger, A. L. 1950. The role of metal ions in enzyme systems. Physiological Reviews 30:393-429.

Lin, A. H. Y. and W. L. Williams. 1966. Growth factors for Lactobacillus bulgaricus GS. The Journal of Nutrition 88:323-330.

Losick, R. and C. Gilvarg. 1966. Effect of α-acetylation on utilization of lysine oligopeptides in Escherichia coli. The Journal of Biological Chemistry 241:2340-2346.

Lucas, G. H. W. 1924. The fractionation of bios, and comparison of bios with vitamins B and C. The Journal of Physical Chemistry 28:1180-1200.

MacLeod, R. A. 1951. Further mineral requirements of Streptococcus faecalis. Journal of Bacteriology 62:337-345.

MacLeod, R. A. and E. E. Snell. 1947. Some mineral requirements of the lactic acid bacteria. The Journal of Biological Chemistry 170:351-365.

Maddox, I. S. and J. S. Hough. 1970. Proteolytic enzymes and autolysing brewers' yeast. In: European Brewery Convention. Proceedings of the 12th Congress, Interlaken, 1969. Amsterdam, Elsevier Publishing Company. p. 315-325.

Markovitz, A. and D. Steinberg. 1957. A method for the determination of peptides in the presence of free amino acids. The Journal of Biological Chemistry 228:285-293.

Mayshak, J. et al. 1966. Inhibition and transport kinetic studies involving L-leucine, L-valine, and their dipeptides in Leuconostoc mesenteroides. Archives of Biochemistry and Biophysics 113:189-194.

McNutt, W. S. and E. E. Snell. 1950. Pyridoxal phosphate and pyridoxamine phosphate as growth factors for lactic acid bacteria. The Journal of Biological Chemistry 182:557-567.

Meeham, D. K. 1971. Lipids. In: Wheat chemistry and technology, ed. by Y. Pomeranz. St. Paul, American Association of Cereal Chemists, Incorporated. p. 393-451.

Merrifield, R. B. and D. W. Woolley. 1958. The synthesis of peptides with strepogenin activity. The Journal of the American Chemical Society 80:6635-6639.

Merrifield, R. B. 1958. Competitive inhibition of a strepogenin-active peptide by related peptides. The Journal of Biological Chemistry 232:43-54.

Metcalf, D., G. J. Hucker and D. C. Carpenter. 1946. A growth factor in certain vegetable juices. Journal of Bacteriology 51:381-384.

Mitchell, H. K., E. E. Snell and R. J. Williams. 1941. The concentration of "folic acid". The Journal of the American Chemical Society 63:2284.

Möller, E. F. 1939. Das Wuchsstoffsystem der Milchsäuerbakterien. Hoppe-Seyler's Zeitschrift für Physiologische Chemie 260: 246-256.

Moore, S. and W. H. Stein. 1963. Chromatographic determination of amino acids by use of automatic recording equipment. In: Methods of enzymology, ed. by S. P. Colowick and N. O. Kaplan. Vol. 6, New York, Academic Press. p. 819-831.

Morris, G. K. and W. L. Williams. 1965. Inhibition of growth of Lactobacillus bulgaricus by purine deoxyribonucleotides. Journal of Bacteriology 90:715-719.

Ng, H. 1972. Factors affecting organic acid production by sourdough (San Francisco) bacteria. Applied Microbiology 23: 1153-1159.

Nimmo-Smith, R. H., J. Lascelles and D. D. Woods. 1948. The synthesis of "folic acid" by Streptobacterium plantarum and its inhibition by sulphonamides. The British Journal of Experimental Pathology 29:264-281.

Ochoa, S. and Y. Kaziro. 1965. Carboxylases and the role of biotin. In: Comprehensive biochemistry, ed. by M. Florkin and E. H. Stotz. Vol. 16, Amsterdam, Elsevier Publishing Company. p. 210-249.

Owen, E. C. and J. Pringle. 1970. Inhibition of growth of Lactobacillus casei by tungstate and its reversal by molybdate. (Abstract) Proceedings of the Biochemical Society. The Biochemical Journal 119:23p-24p.

Payne, J. W. and C. Gilvarg. 1968a. The role of the terminal carboxyl group in peptide transport in Escherichia coli. The Journal of Biological Chemistry 243:335-340.

_____ 1968b. Size restriction on peptide utilization in Escherichia coli. The Journal of Biological Chemistry 243: 6291-6299.

_____ 1971. Peptide transport. In: Advances in enzymology, ed. by A. Meister. Vol. 35, New York, Interscience Publishers. p. 187-244.

Pogo, A. O. et al. 1967. Modification of ribonucleic acid synthesis in nuclei isolated from normal and regenerating liver: some effects of salt and specific divalent cations. Proceedings of the National Academy of Sciences, U. S. A. 57:743-750.

Pittman, K. A. and M. P. Bryant. 1964. Peptides and other nitrogen sources for growth of Bacteroides ruminicola. Journal of Bacteriology 88:401-410.

Pittman, K. A., S. Lakshmanan and M. P. Bryant. 1967. Oligopeptide uptake by Bacteroides ruminicola. Journal of Bacteriology 93:1499-1508.

Pyke, M. 1958. X. The technology of yeast. In: The chemistry and biology of yeasts, ed. by A. H. Cook. New York, Academic Press. p. 535-586.

Rogosa, M. 1944. Microbiological method for the quantitative determination of small quantities of potassium. The Journal of Biological Chemistry 154:307-308.

Rogosa, M. et al. 1953. Species differentiation of oral lactobacilli from man including descriptions of Lactobacillus salivarius nov spec and Lactobacillus cellobiosus nov spec. Journal of Bacteriology 65:681-699.

Rogosa, M., J. G. Franklin and K. D. Perry. 1961. Correlation of the vitamin requirements with cultural and biochemical characters of Lactobacillus spp. The Journal of General Microbiology 25:473-482.

Rothstein, A. and A. D. Hayes. 1956. The relationship of the cell surface to metabolism. XIII. The cation-binding properties of the yeast cell surface. Archives of Biochemistry and Biophysics 63:87-99.

Sarett, H. P. 1947. Interrelationship between p-aminobenzoic acid and pteroylglutamic acid as growth factors for lactobacilli. The Journal of Biological Chemistry 171:265-272.

Saunders, R. M., H. Ng and L. Kline. 1972. The sugars of flour and their involvement in the San Francisco sour dough French bread process. Cereal Chemistry 49:86-91.

Seifter, S. et al. 1950. The estimation of glycogen with the anthrone reagent. Archives of Biochemistry and Biophysics 25:191-200.

Sheard, B. et al. 1967. Proton magnetic relaxation studies of the binding of manganese ions to E. coli ribosomes. Journal of Molecular Biology 28:389-402.

Shorb, M. S. 1948. Activity of vitamin B_{12} for the growth of Lactobacillus lactis. Science 107:397-398.

Silver, S., P. Johnseine and K. King. 1970. Manganese active transport in Escherichia coli. Journal of Bacteriology 104: 1299-1306.

Smith, R. L., E. G. Archer and F. W. Dunn. 1970. Uptake of [^{14}C] - labeled tri-, tetra-, and pentapeptides of phenylalanine and glycine by Escherichia coli. The Journal of Biological Chemistry 245:2967-2971.

Snell, E. E. 1958. Chemical structure in relation to biological activities of vitamin B_6. In: Vitamins and hormones, advances in research and applications, ed. by R. S. Harris, G. F. Marrion and K. V. Thimainin. Vol. 16, New York, Academic Press. p. 77-125.

Snell, E. E., B. M. Guirard and R. J. Williams. 1942. Occurrence in natural products of a physiologically active metabolite of pyridoxine. The Journal of Biological Chemistry 143:519-530.

Snell, N. and J. C. Lewis. 1953. Nutritional studies with Lactobacillus fermenti. Journal of Bacteriology 65:671-677.

Snell, E. E. and H. K. Mitchell. 1941. Purine and pyrimidine bases as growth factors for lactic acid bacteria. Proceedings of the National Academy of Sciences, U. S. A. 27:1-7.

Snell, E. E. and W. H. Peterson. 1940. Growth factors for bacteria. X. Additional factors required by certain lactic acid bacteria. Journal of Bacteriology 39:273-285.

Snell, E. E. and A. N. Rannefeld. 1945. The vitamin B_6 group. III. The vitamin activity of pyridoxal and pyridoxamine for various organisms. The Journal of Biological Chemistry 157:475-489.

Snell, E. E., F. M. Strong and W. H. Peterson. 1937. Growth factors for bacteria. VI. Fractionation and properties of an accessory factor for lactic acid bacteria. The Biochemical Journal 31:1789-1799.

Soska, J. 1966. Growth of Lactobacillus acidophilus in the absence of folic acid. Journal of Bacteriology 91:1840-1847.

Sprince, H. and D. W. Woolley. 1944. Relationship of a new growth factor required by certain hemolytic streptococci to growth phenomena in other bacteria. The Journal of Experimental Medicine 80:213-217.

Sriranganathan, N. et al. 1973. Cytological and deoxyribonucleic acid-deoxyribonucleic acid hybridization studies on Lactobacillus isolates from San Francisco sourdough. Applied Microbiology 25:461-470.

Stamer, J. R., M. N. Albury and C. S. Pederson. 1964. Substitution of manganese for tomato juice in the cultivation of lactic acid bacteria. Applied Microbiology 12:165-168.

Stokes, J. L., A. Larsen and M. Gunness. 1947a. Biotin and the synthesis of aspartic acid by microorganisms. Journal of Bacteriology 54:219-230.

_____ 1947b. Biotin and the synthesis of aspartic acid by microorganisms. The Journal of Biological Chemistry 167:613-614.

Strittmatter, C. F. 1959. Electron transport to oxygen in lactobacilli. The Journal of Biological Chemistry 234:2789-2793.

Sugihara, T. F., L. Kline and L. B. McCready. 1970. Nature of the San Francisco sour dough French bread process. II. Microbiological aspects. The Bakers Digest 44:51-57. Apps

Sugihara, T. F., L. Kline and M. W. Miller. 1971. Microorganisms of the San Francisco sour dough bread process. I. Yeasts responsible for the leavening action. Applied Microbiology 21:456-458.

Tritsch, G. L. and O. Grahl-Nielsen. 1969. The structure of growth controlling peptides for mammalian cells in axenic culture. Biochemistry 8:1816-1821.

Tritsch, G. L. and D. W. Woolley. 1960. The synthesis of L-leucyl-L-valyl-L-cysteinylglycyl-L-glutamyl-L-arginine, an insulin fragment with strepogenin activity. The Journal of the American Chemical Society 82:2787-2793.

Wakil, S. J. 1965. Mechanism of fatty-acid biosynthesis. Science 150:388.

Weinberg, E. D. 1970. Biosynthesis of secondary metabolites: roles of trace metals. Advances in Microbial Physiology 4:1-44.

Weinman, D. E., G. K. Morris and W. L. Williams. 1964. Unidentified growth factor for a lactic acid bacterium. Journal of Bacteriology 87:263-269.

Weissbach, H., A. Peterkofsky and H. A. Barker. 1965. The cobamide coenzymes. In: Comprehensive biochemistry, ed. by M. Florkin and E. H. Stotz. Vol. 16, Amsterdam, Elsevier Publishing Company. p. 189-209.

Wildiers, E. 1901. Nouvelle substance indispensable au développement de la levûre. La Cellule 18:311-333.

Williams, R. J. and R. T. Major. 1940. The structure of pantothenic acid. Science 91:246.

Williams, R. J. and R. R. Roehm. 1930. The effect of antineuritic vitamin preparations on the growth of yeasts. The Journal of Biological Chemistry 87:581-590.

Williams, V. R. and E. A. Fieger. 1946. Oleic acid as a growth stimulant for Lactobacillus casei. The Journal of Biological Chemistry 166:335-343.

Woolley, D. W. 1941. A new growth factor required by certain hemolytic streptococci. The Journal of Experimental Medicine 73:487-492.

Woolley, D. W. et al. 1955. Strepogenin activity of synthetic peptides related to oxytocin. Proceedings of the Society for Experimental Biology and Medicine 89:669-673.

Woolley, D. W. and R. B. Merrifield. 1958. Specificity of peptides. Science 128:238-240.

_____ 1963. Anomalies of the structural specificity of peptides. Annals of the New York Academy of Sciences 104: 161-171.

Zlataroff, A. and D. Kaltschewa. 1936. Der Einfluenz einiger Metallsalze auf die Milchsäuergarung. Biochemische Zeitschrift 284:12-23.