Enzyme Technology

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ENZYME TECHNOLOGY

BOOKS BY HENRY TAUBER

ENZYME CHEMISTRY

A general summary of advances in the chemistry of enzymes.

ENZYME TECHNOLOGY

A practical book dealing with the application of enzymes and useful subjects on fermentation.

PREFACE

This volume presents practical material concerning the role and use of enzymes in industry, their preparation, and their use in medicine. A part of the book deals with the highly instructive and commercially significant preparation of organic compounds by fermentation. The view that molds and almost all bacteria are only destructive agents which burn up sugars and offer nothing in return is out of date. In recent years a large variety of microorganisms have been put to work on a factory scale and are now producing 24 hours a day. Thus one product of fermentation, sorbose, is an intermediary in the manufacture of vitamin C, another, butylene glycol, may be used in the synthesis of rubber, and a fermentation product of yeast, *l*-acetylphenylcarbinol, is employed in the synthesis of *l*-ephedrine. One mold product, penicillin, is being applied therapeutically with great success. For the production of these substances definite substrates and conditions are required.

Many industrial problems have been solved by the proper application of enzyme chemistry; others await improvement or solution. Indeed the fields for exploration offered by catalytic microorganisms and practical enzymology are almost unlimited.

A series of quantitative methods for the determination of enzymes and vitamins has also been included in the book because of their usefulness in research and control work.

For detailed descriptions of enzymes, such as their activation and inactivation, their mechanism of action, their chemical nature, the crystalline enzymes, etc., the reader is referred to a general text on enzymes such as the author's " Enzyme Chemistry." A duplication of such subjects has been purposely avoided.

I wish to express my gratitude to Dr. A. K. Balls of the Bureau of Agricultural Chemistry and Engineering, United States Department of Agriculture; Dr. A. C. Ivy of the Northwestern University Medical School; Dr. Z. I. Kertesz of the New York Agricultural Experiment Station; Dr. P. J. Kolachov of Joseph E. Seagram and Sons, Inc.; Dr. S. Laufer of the Schwarz Laboratories, Inc.; Dr. G. D. McLaughlin of the B. D. Eisendrath Memorial Laboratory, who very kindly read parts of the manuscript and offered valuable suggestions.

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HENRY TAUBER

Long Island City, N. Y.
August, 1943

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CHAPTER I

YEAST: PRODUCTION AND UTILIZATION AND THE ENZYME SYSTEMS

THE MANUFACTURE OF COMPRESSED YEAST

In the United States 230,000,000 pounds of yeast are manufactured per year (1).[1] Most of it is used in the baking, brewing, and alcohol industries. Some of the yeast is employed as a source of vitamins; some, in the manufacture of soluble proteins; some, in the manufacture of glutathione, nucleic acid, enzymes, etc. Extracts of yeast are used in the preparation of media for bacteria.

Commercially the most important yeasts are beer yeasts, distillers' yeasts, bakers' yeasts, and wine yeasts. The yeast manufacturer is interested only in the type called *Saccharomyces cerevisiae*. This group contains a large number of strains. The isolation and preservation of the proper strain are of great importance.

The Molasses-Ammonia Process. The molasses-ammonia process originated by Hayduck is at present the most widely used method for the manufacture of compressed yeast. In this process, molasses (cane or sugar beet), mineral salts, and ammonia are the principal nutrients. The *p*H is continuously controlled. Very dilute wort is employed during the first phase of growth and the concentrated wort is continuously added at such a rate that any alcohol that may form is assimilated during the growth of yeast. The nutrients are supplied in such amounts that they closely parallel the yeast growth rate. Although only small amounts of alcohol are formed by this process, the yield of yeast is very high.

The most favorable *p*H for yeast growth is between *p*H 3 and *p*H 4.5. If an acid *p*H is maintained, bacterial growth may be completely eliminated. Sulfuric acid and ammonium hydroxide or another alkali are used to adjust the *p*H of the wort. The ammonia is continuously used up by the growing yeast and, as a result, the mash becomes more and more acid and must be neutralized.

The temperature is controlled by cooling coils placed inside the containers. The optimum temperature varies with the strain of yeast

[1] Numbers in parentheses refer to items in References at end of chapter.

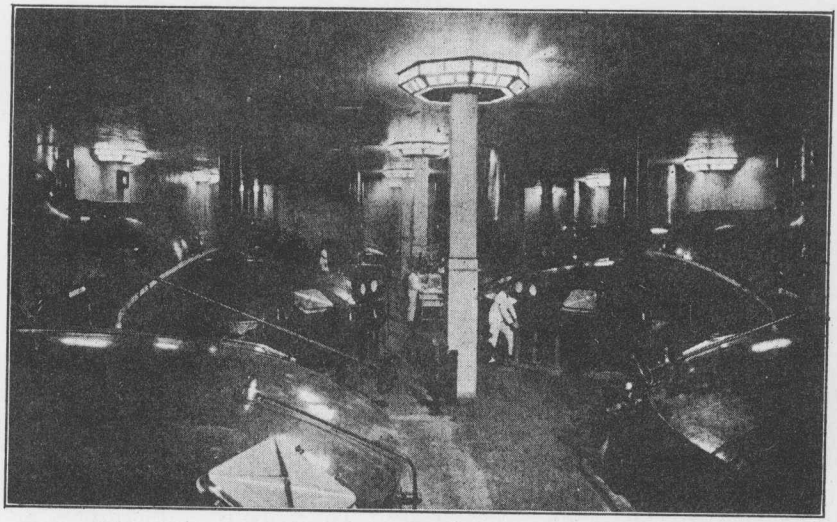


FIG. 1. Fermenting room.

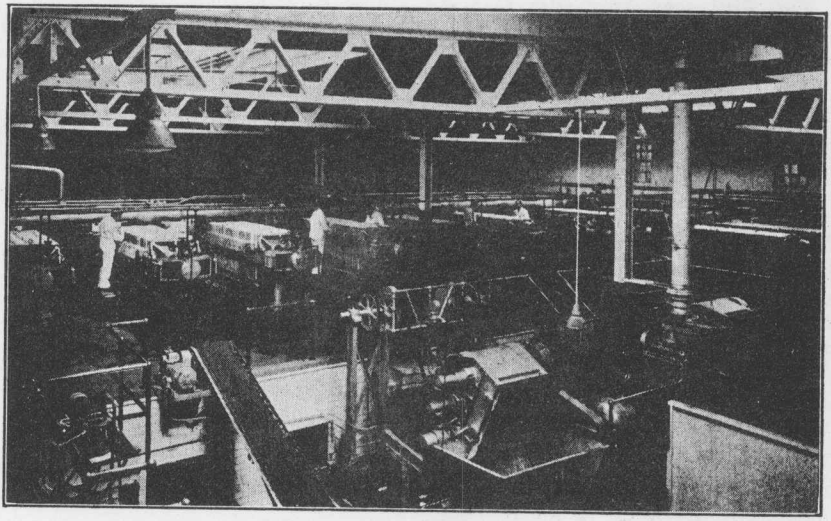


FIG. 2. Filter pressing room.

employed. When growth is completed the yeast is separated from the wort by filtration or centrifugation and is washed and pressed. Flour may be added to the yeast to aid in handling.

The following is a recently improved version of yeast manufacture by the molasses-ammonia process (2).

One thousand three hundred and twenty-three pounds of yeast are brought to an acid content of 2° Balling, and the yeast is allowed to stand for 1 hour. Then 8000 gallons of water are placed in a container of 16,000 gallons capacity. The acidified pitching yeast is added to the water and is well mixed. The wort has a concentration of 1° Balling, and 20 cc. of the wort require 0.35 cc. of a $N/10$ sodium hydroxide solution to become neutral to litmus. The wort, which is still free of molasses, is aerated with 800,000 gallons of air per hour and is then mixed gradually during a period of 11 hours with 7711 pounds of molasses. Care is taken that during the first 9 hours the wort is always slightly acid.

Nitrogen and phosphorus are supplied in the form of 330 pounds of superphosphate, 99 pounds of ammonium persulfate, and 25 gallons of 25 per cent ammonia. After the addition of the total quantity of nitrogen and phosphorus (after 9 hours), 0.4 cc. of a $N/10$ sodium hydroxide solution is required to neutralize 20 cc. of wort, using litmus as an indicator. Maintenance of strong aeration causes the acidity to drop and, after 10 hours, only 0.25 cc. of a $N/10$ sodium hydroxide solution is necessary to neutralize the same volume of wort after all the molasses is added. After 11 hours the wort is neutral to litmus. Aeration is continued for a short time. It is gradually reduced and discontinued after 2½ hours more. The yeast is now matured and the wort is free of sugar and acid. Eleven thousand ninety-five pounds of wort of 3.4° Balling are obtained. The yield of yeast is 5458 pounds which, after deducting 1323 pounds used for pitching, corresponds to 53 per cent of the molasses used. The alcohol yield is 132 gallons or 12 per cent of the weight of molasses used.

Figures 1 and 2 show two main phases of the manufacture of compressed yeast (by courtesy of Dr. C. N. Frey of the Fleischmann Laboratories, Inc.).

Production of "Galac" Yeast. In order that yeast should be able to grow on a medium containing certain substances such as sulfites or other than the usual sugars, the yeast must first be acclimatized to the abnormal conditions. According to Nilsson (3), bottom yeast may be readily "trained" to ferment galactose when cultivated on the following medium:

Washed and pressed bottom yeast	500 grams
Galactose	100 "
KH_2PO_4	45 "
Na_2HPO_4	5 "
Yeast extract	250 cc.
Water	5000 "

The yeast is grown at 25°C. Every day for five days 100 grams of galactose are added. By the sixth day the galactose-fermenting mechanism is considerably developed and galactose is rapidly fermented by the yeast. This yeast may be dried at room temperature without loss of activity.

Other Methods of Yeast Manufacture. In the Heijkenskjöld process (4) sulfite liquor and a small amount of molasses are used as the raw material. In the Scholler-Tornesch process (5) wood sugar and added mineral salts are employed for the manufacture of fodder yeast.

For a discussion of the large scale production of compressed yeast as practiced in Germany, see reference 6. Other processes for the manufacture of compressed yeast have recently been described in detail by Walter (7). A useful monograph on pure yeast culture systems has been published by Laufer and Schwarz (8). See also Chapter II. Table I shows yeast yields obtained when grown in three different media on a laboratory scale (9).

TABLE I

YIELDS OF DRY YEAST GROWN IN VARIOUS MEDIA*

Type of yeast	Grain medium %	Molasses-salts medium %	Glucose-salts medium %
Bakers' yeast A	24.3	34.6	18.0
Bakers' yeast B	42.5	33.6	34.3
Brewers' yeast A	34.6	42.7	29.0
Brewers' yeast A (autoclaved medium)	32.2	...	...
Saccharomyces logos	33.1	28.0	21.4
Willia anomala	21.4	28.6	11.4
Endomyces vernalis	40.9	33.6	30.5

* Dry yeast is based on glucose fermented.

Culture Media for Yeast. Yeast may be grown in all glucose-containing fruit juices, in beer wort, or in the following synthetic media after they have been sterilized at 15 pounds pressure for 15 minutes (7).

A.	Dextrin and sugar....	100.00 grams		$MgSO_4$.....	0.1 grams
	$(NH_4)_2SO_4$.....	4.75 "		$CaSO_4$.....	0.1 "
	KH_2PO_4.....	0.75 "		Water to 1 liter.	
B.	Sugar.....	15.0 per cent	KH_2PO_4	0.2 per cent	$CaCO_3$.....	0.1 per cent
	Water.....	84.0 " "	$MgSO_4$	0.1 " "	$(NH_4)K_2PO_4$	0.5 " "
C.	Sugar.....	15.0 per cent	Water..	83.5 per cent	$MgSO_4$.....	0.2 per cent
	Asparagine	0.7 " "	KH_2PO_4	0.5 " "	$CaCO_3$.....	0.1 " "
D.	Sugar.....	15.0 per cent	Water..	83.5 per cent	$MgSO_4$.....	0.1 per cent
	Peptone Witte...	0.5 " "	KH_2PO_4	0.5 " "	$CaCO_3$.....	0.1 " "

If solid transparent media are desired 1 per cent by weight of agar or gelatin should be added to the above solutions before sterilization.

THE RELATION OF GROWTH SUBSTANCES TO YEAST

There are a number of organic compounds acting as food accessories for certain microorganisms. Very small quantities, however, are sufficient when added to their usual carbon source and inorganic salt supply. β-Alanine, for example, is most effective at a concentration of 1:10,000,000, and is still active at a concentration of 1:200,000,000. Large concentrations may have an inhibitory effect.

The requirement for growth substance depends on the type of microorganism, the composition of the medium and environmental factors (10–12). Lesh, Underkofler, and Fulmer (12) showed that magnesium sulfate stimulates bios activity of certain types of *Saccharomyces cerevisiae* yeasts, but not of other strains of the same group. According to Schultz, Atkin and Frey (13), *thiamin* stimulates only some strains of *S. cerevisiae,* whereas others are inhibited. Schopfer (14) reported that a red yeast (*Rhodotorula rubra*), which is known to require thiamin, in reality needs only *pyrimidine,* whereas *Mucor ramanninanus* requires only *thiazole.*

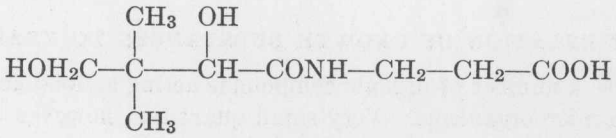
In 1901 Wildier gave the name " bios " to a hypothetical organic substance that stimulated yeast growth. Although Wildier's conception of bios is incorrect, it led the way to important discoveries by others.

The bios complex is soluble in water and 80 per cent alcohol. It is insoluble in absolute alcohol and ether. It dialyzes readily. Bios may be separated into two fractions by treatment with alcoholic barium hydroxide solution. The fraction which forms an insoluble barium salt was named " bios I," whereas the residual solution was called " bios II " (15), which may be further separated into various fractions.

Inositol. It was shown that bios I was identical with the optically inactive inositol (16). Inositol itself is generally without effect, but

increases the action of bios constituents (17). Not all yeasts respond to inositol, however (18). In plants such as barley, inositol is present as the phosphoric acid ester, phytin.

Pantothenic Acid. Williams and associates (19, 20) have separated bios II with Fuller's earth into two fractions: one, which is replaceable by thiamin, and another fraction, named " pantothenic acid," which is not replaceable by thiamin. β-Alanine is a part of the pantothenic acid molecule (21). The growth-stimulating action of this amino acid upon yeast is well established. On the basis of this work Merck research chemists obtained pantothenic acid synthetically (22, 23). It has the following structural formula:



PANTOTHENIC ACID

(α,γ-Dihydroxy-β,β-dimethylbutyryl-β-alanide)

Pantothenic acid is formed by condensation of α-hydroxy-β, β-dimethyl-γ-butyrolactone (a known synthetic product), with β-alanine. Thiamin and inositol or a mixture of both increases the action of the acid on certain yeasts (24).

Pantothenic acid is a highly active growth promoter. A concentration of 1:50,000 increases the growth of yeast five times over that of the control experiment (20). Some yeasts are stimulated by pantothenic acid only in the presence of thiamin or β-alanine. Certain yeasts that cannot normally produce this acid can do so when β-alanine is the only nitrogen supply in the medium (25). Liver and rice bran are very good sources of pantothenic acid, and appear to affect the growth of higher plants and animals (26, 27).

Biotin and Other Growth Factors. This bios factor may be obtained by fractionating bios with charcoal. The charcoal adsorbs bios II. It may be eluted with dilute ammonium hydroxide and acetone (28, 29). The unadsorbed part has been named bios III.

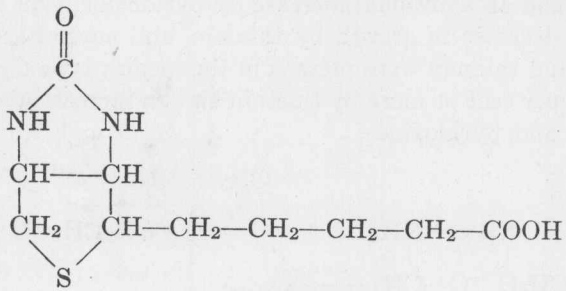
Biotin and pantothenic acid are chick antidermatitis factors. They are not identical, however (18).

Biotin is the most active biological substance. One part in fifty billion may be distinctly detected in the growth of yeast. Traces of it are present in all important animal organs and body fluids, as well as in many plants (30). Lampen, Bahler, and Peterson (31) analyzed the biotin content of a series of biological materials. Extraction at an

acid *p*H was much more effective than water. The following were found to be the three best sources: beef kidney containing 2500, pork liver 2000, and brewers' yeast 830 millimicrograms of biotin per gram of dry matter. Other good sources are malt rootlets (30) and leaves of the birch (33).

Chemical Nature of Biotin. Investigators at Cornell University Medical College and at the School of Medicine, Western Reserve University (34), have found in a cooperative study that the three factors known as biotin, *rhizobia* (the growth and respiration factor), and vitamin H (the anti-egg-white-injury factor) are one and the same substance.

The structural formula of biotin has recently been elucidated by Du Vigneaud and associates (35). It contains a five-membered sulfur ring with a valeric acid side chain combined with the carbon alpha to the sulfur. The sulfur ring is attached to a cyclic urea structure:



BIOTIN

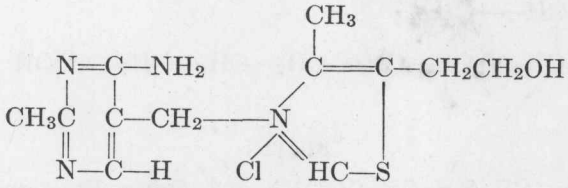
β-Alanine ($CH_2NH_2CH_2COOH$) and Other Fractions of Bios. Bios II, when treated with charcoal, may be separated into two components (36). The charcoal adsorbate which is liberated by shaking with an aqueous solution of acetone and ammonia, has been named "bios IIB," whereas the unadsorbed fraction has been called "bios IIA." Bios IIA contains β-alanine and leucine (37). In the presence of sugar, inorganic salts, and 5 mg. of inositol per liter, the growth of some yeasts was stimulated by a concentration of 1:12,000,000 of β-alanine. With the addition of aspartic acid, the effect was increased (38). According to Nielsen and Hartelius (39), β-alanine was toxic in the absence of asparagine or aspartic acid.

Nielsen and Dagys (40) reported that β-alanine, in order to function as a growth factor for yeast, requires biotin, thiamin, asparagine, and glutamic acid. For the last two items may be substituted succinic, citric, tartaric, or malic acid. The action of one of these acids increases the functioning of β-alanine tenfold.

Heating glucose and ammonium tartrate solutions together produced a growth substance for yeast which acted in a way similar to β-alanine. It is suggested that β-alanine itself may form during the reaction.

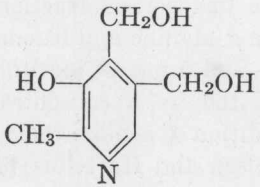
The growth-promoting action is not a property of the amino group in the *beta*-position. β-Phenyl-β-alanine, β-aminobutyric acid, glycine, δ-aminovaleric acid, or ε-aminocaproic acids could replace β-alanine (41). Among 31 amino acids studied only β-alanine, lysine, arginine, methionine, aspartic acid, asparagine, and glutamic acid stimulated growth. β-Alanine was the most effective stimulant (42).

Pyridoxine (Vitamin B_6) and Thiamin. Williams, Eakin, and Snell investigated three strains of *S. cerevisiae* as to their pyridoxine requirement in the presence of inositol, pantothenic acid, and biotin, and found that it is relatively unimportant. Schultz, Atkin, and Frey (41, 43), studied forty-four strains of *S. cerevisiae* and *S. carlsbergensis* and divided them into three groups: type *A*, showing growth increase by thiamin and an additional increase by pyridoxine, type *B*, showing 50 per cent decrease in growth by thiamin, and normal growth when pyridoxine and thiamin were present in the media; type *C*, with a decrease of 50 per cent or more by thiamin and an increase above normal by thiamin and pyridoxine.



THIAMIN (VITAMIN B_1)

(2 Methyl-5-[4 methyl-5-β-hydroxyethyl-thiazolium chloride]methyl-6-amino-pyrimidine)



PYRIDOXINE

(2 Methyl-3 hydroxy-4,5-di-[hydroxymethyl]-pyridine)

Thus pyridoxine is required by some of the yeasts and acts only in the presence of thiamin. The mechanism of the functions of the panto-

thenic acid, inositol, biotin, β-alanine, and pyridoxine are not yet known.

Assay methods for the various growth substances have been described by Williams and coworkers (44).

TABLE II

COMPOSITION OF YEAST

Crude proteins	46.74 per cent
Fat	1.61 " "
Carbohydrates	35.37 " "
Ash	7.87 " "
Crude fiber	8.41 " "

TABLE III

AMINO ACID CONTENT OF YEAST

Cystine	0.49 per cent
Arginine	3.50 " "
Trypotophan	2.67 " "
Histidine	1.38 " "
Tyrosine	4.11 " "
Lysine	4.50 " "

Yeast also contains about 0.2 per cent of glutathione, on a dry basis.

The Composition of Yeast. Table II shows a typical analysis of beer yeast substance (45).

Yeast is a complete protein since it contains all the essential amino acids (46). (See Table III.)

Phosphorus Compounds of Yeast. Phosphates play a very important role in the metabolism of the yeast cell. Addition of phosphates results in a considerable increase in fat and carbohydrate storage. The following phosphoric acid esters have been isolated: hexosemonophosphate, hexosediphosphate, trehalose monophosphate, phosphoglyceric acid, phosphoglycerol, phosphoglyceraldehyde, dihydroxyacetone phosphate, dihydroxyacetone diphosphate, phosphopyruvic acid, nucleic acid, nucleoproteins, coenzyme I, coenzyme II, riboflavin monophosphates, diphosphothiamin, and phospholipides.

Table IV shows the relative concentrations of various phosphorus compounds (47), and Table V represents the composition of yeast ash. (For an excellent review concerning the mineral metabolism of yeast see reference 48.)

TABLE IV

PHOSPHORUS COMPOUNDS OF ENGLISH BREWERS' YEAST
(Milligrams of phosphorus per gram of yeast)

Compound	English brewers' yeast
Total phosphorus	3.25
Orthophosphate	1.37
Pyrophosphate	0.68
Organic phosphate	1.17
Hexosediphosphate	0.38
Hexosemonophosphate	0.72
Nucleic acid	0.07

TABLE V

ANALYSIS OF YEAST ASH (PERCENTAGE)

Ash constituent	Top yeast*	Bakers' yeast†
P_2O_5	52.3	54.5
K_2O	35.4	36.5
Na_2O	0.06	0.7
MgO	4.8	5.2
CaO	1.56	1.4
SiO_2	1.1	1.2
SO_3	0.41	0.5
Cl	....	trace
FeO	0.43	trace

* Fulmer *et al.* (1928).
† Frey (1930).

YEAST AS A FOOD SUPPLEMENT

In addition to its high protein, fat, and mineral salts content, yeast contains large quantities of thiamin, riboflavin, nicotinic acid, provitamin D, pantothenic acid, pyridoxine, biotin and *p*-aminobenzoic acid.

Stone (49) suggested a ration of brewers' yeast for the United States and British forces. Such rations have been in use by some European troops. In Britain surplus brewers' yeast is being put to many uses. According to Stone, " It goes to make ' meat extracts,' packet gravies and soups. The demand for ' marmite ' and similar yeast products far exceeds the supply. Yeast also is incorporated in poultry foods and has been found suitable to supplement the oat ration of horses if added to the extent of one-eighth of the oat ration, with a small addition of salt. It has been found that irradiated yeast increases the milk production of cows and that the milk from cows so fed has an increased fat and vitamin D content. This diet has been found to effect a cure in the case of cows suffering from tuberculosis." This paper contains