resemble ginger ale to darker products similar to hydrolyzed vegetable protein. The enzyme filtrates can be vacuum-concentrated and then be standardized to the desired potency.

Precipitated purified powdered materials are either sold as concentrates on the basis of their potency, or diluted with various agents to standard activities. For this purpose, the ground enzyme concentrate powders are assayed for their enzyme potency, and are then uniformly blended with necessary amounts of edible diluents consistent with their end uses. Examples of the edible diluents are: dextrose, lactose, sucrose, starch, flour, salts, gelatin, and casein. Where the food product is filtered after enzyme treatment, the diluents may include diatomaceous earth. Buffers and other salts, such as citrates or phosphates, calcium sulfate, etc., are also used in some instances in order to maintain favorable pH conditions, enzyme activity, and stability. Frequently it is necessary to dilute the enzyme concentrate beyond the point normally required for standardization, because for certain uses the small amount of enzyme required cannot be handled by the customer with accuracy.

Summary

Microbial enzymes have a long history of safe usage in food products.

The procedures used in their commercial production involve combinations of an appropriate, potent strain of organism, the right medium composition, and the correct conditions for fermentation and enzyme recovery. Continuous rigid control of all steps of the operations is necessary to assure maximum enzyme yields, and reproducibility, safety, and quality of the enzyme products.

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Literature Cited

(1) American Pharmaceutical Association, Washington, D. C., "National Formulary XI," 1960.
(2) Anson, M. L., *J. Gen. Physiol.* **22,** 79 (1938).
(3) Association of Official Agricultural Chemists, Washington, "Official Methods of Analysis," 9th ed., 1960.
(4) Beckhorn, E. J., *Wallerstein Lab. Commun.* **82,** 201 (1960).
(5) Breed, R. S., Murray, E. G. D., Smith, N. R., "Bergey's Manual of Determinative Bacteriology," Williams & Wilkins, Baltimore, 1957.
(6) Colowick, S. P., Caplan, N. O., "Methods in Enzymology," Academic Press, New York, 1955–1962.
(7) Gocar, M., *Petit J. Brasseur* **62,** 612 (1954).
(8) Hoogerheide, J. C., in "Industrial Fermentations," L. A. Underkofler, R. J. Hickey, eds., Vol. **2,** Chap. 3, Chemical Publishing Co., New York, 1954.
(9) Howard, K. L., *Biscuit Cracker Baker* **45,** 30 (1956).
(10) Kirsch, D., *J. Biol. Chem.* **108,** 421 (1935).
(11) Koch, R. B., Ferrari, C. G., *Cereal Chem.* **32,** 254 (1955).
(12) Northrop, J. H., *J. Gen. Physiol.* **15,** 29 (1931).
(13) Oshima, K., Church, M. B., *J. Ind. Eng. Chem.* **15,** 67 (1923).
(14) Prescott, S. D., Dunn, C. G., "Industrial Microbiology," 3rd ed., Chap. 41, McGraw-Hill, New York, 1959.
(15) Reed, G., "Encyclopedia of Chemical Technology," R. E. Kirk, D. F. Othmer, eds., 1st suppl. vol., Interscience Encyclopedia, New York, 1957.
(16) Sandstedt, R. M., Kneen, E., Blish, M. J., *Cereal Chem.* **16,** 712 (1939).
(17) Smythe, C. V., *Economic Botany* **5** (2), 126 (1951).
(18) Takamine, J., *J. Soc. Chem. Ind.* **17,** 118 (1898).
(19) Takamine, J., *J. Ind. Eng. Chem.* **6,** 824 (1914).
(20) Thom, C., Raper, K. B., "Manual of the Aspergilli," Williams & Wilkins, Baltimore, 1945.
(21) Thomas, R., *Australian J. Biol. Sci.* **9,** 159 (1956).
(22) Tietz, N. W., Borden, T., Stapleton, J. D., *Am. J. Clin. Pathol.* **31,** 148 (1959).
(23) Underkofler, L. A., in "Industrial Fermentations," L. A. Underkofler, R. J. Hickey, eds., Vol. **2,** Chap. 2, Chemical Publishing Co., New York, 1954.
(24) Underkofler, L. A., Barton, R. R., Rennert, S. S., *Appl. Microbiol.* **6,** 212 (1958).
(25) U. S. Dept. Agriculture, Tech. Bull. **1024** (1950).
(26) *Ibid.*, **1053** (1952).
(27) U. S. Pharmacopoeial Convention, "Pharmacopoeia of the U. S. A.," 16th ed., Easton, Pa., 1960.
(28) Waksman, S. A., Davison, W. C., "Enzymes. Properties, Distribution, Methods, and Applications," Williams & Wilkins, Baltimore, 1926.
(29) Wallerstein, L., *Ind. Eng. Chem.* **31,** 1218 (1939).

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FOOD YEAST PROTEIN

Amino Acid Composition of Yeast Grown on Different Spent Sulfite Liquors

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Advances in sulfite pulp mill technology and changes in standards for paper products generally have altered the composition of spent sulfite liquor so much as to modify the performance of the food yeast, *Candida utilis*, grown continuously in it. The complexity of spent sulfite liquor composition varies with the kind of wood selected for pulping, the base ion used to form the cooking acid, and the degree of cooking (*14, 15*). During pulping, hardwoods (beech, aspen, or poplar) generally yield more sugars and acetic acid than softwoods (spruce or balsam). Hardwood spent liquors contain up to 3% sugar (liquor basis), mainly xylose. In softwood liquors the sugar content is near 2%, three fourths of it being hexose (mainly mannose). For the preparation of the cooking acid, calcium, magnesium, sodium, or ammonium base may be used to absorb the sulfur dioxide.

In the operations observed here, numerous changes in pulping conditions were made, including eventually a shift from calcium base to ammonium base. Spent sulfite liquors (SSL) shunted to the food yeast propagators during this period differed greatly in content of available sugars and nonsugar carbon. Wide fluctuations in yeast yield occurred. The irregularities prompted some concern for product quality, particularly in the yeast protein which makes up more than half the dry yeast weight. Of the several factors examined in this study, only the results of determinations of

Food yeasts (*Candida utilis*), produced continuously on supplemented calcium- and ammonium-base spent sulfite liquor, were assayed at intervals over a 9-month operating period to determine the uniformity of amino acid composition and the folic acid content. The range of values for 18 amino acids remained narrow throughout the period of major changes in fermentation substrates, and the composition of yeast produced on ammonium-base liquor differed little from that of yeast grown on calcium-base liquor. Folic acid data, obtained over a 3-year period, indicate essentially similar values for *Candida utilis* food yeasts and molasses-grown *Saccharomyces cerevisiae*, but both types of yeast contain only half as much folic acid as stated in earlier reports. The free folic acid contents of each type of food yeast average less than 1 μg. per gram. All folic acid values are well within the permissible levels established by the Food and Drug Administration.

Table I. Yeast Performance on Ammonium- and Calcium-Base Spent Sulfite Liquors

	Calcium Base	Ammonium Base
Sugar in feed, g./l.	20.2–21.7	15.3–20.7
Sugar loading, lb./hr.	939–1081	750–1076
Sugar utilization, %	77.6–89.7	77.5–88.8
Retention time, min.	197–219	182–217
Yeast yield, % on sugar	50.2–68.0	54.2–67.3

amino acids and folic acid are reported here.

The vitamin assays were extended over a 3-year period. Both total and free folic acid content were determined for both types of commercial, primary-grown food yeasts: *Candida utilis* grown on spent sulfite liquors and *Saccharomyces cerevisiae* grown on blended worts of beet and cane molasses.

Procedure

Preliminary test periods were coordinated with pulp mill schedules in order to obtain a fair supply of SSL for the yeast fermentors. After allowing time for yeast adaptation to the test liquors, a 10-day observation period of yeast production on each of the two bases was chosen. Yeast performance was judged primarily by the rate of sugar utilization and the yield of dry yeast solids per unit of total reducing sugar (Table I). Propagations followed established procedures and process control (*9*, *10*). Continuous additions of ammonia, phosphoric acid, and potassium chloride were made at rates based on the reducing sugar content of the feed liquor (*14*). Daily composites of dried yeast produced on each test SSL were combined and assayed for amino acids and compared with data (Table II) obtained chromatographically on similar production samples (*5*, *6*).

Table II. Amino Acid Composition of Food Yeast Grown on Ammonia Spent Sulfite Liquor Following Calcium Spent Sulfite Liquor

(%, protein basis)

	Ca Base[a]		NH_3 Base[a]	
	A	B	A	B
Valine	3.8	6.1	3.0	6.1
Leucine	7.6	7.1	6.2	7.2
Isoleucine	3.8	5.1	3.8	5.7
Threonine	5.4	5.4	5.6	5.4
Methionine	1.1	1.0	0.8	1.0
Phenylalanine	8.6	4.3	7.8	4.1
Tryptophan	2.4	1.3	2.8	1.3
Histidine	1.2	1.9	2.6	1.9
Lysine	4.8	6.6	5.6	6.6
Glycine	3.6	4.8	3.4	5.1
Alanine	5.8	3.4	5.6	3.4
Serine	5.0	5.1	6.4	4.7
Proline	6.0	3.4	6.2	3.6
Tyrosine	6.2	3.2	4.8	3.3
Aspartic acid	9.2	4.2	9.8	4.3
Glutamic acid	15.6	14.9	17.0	14.9
Arginine	5.4	5.4	5.2	5.3

[a] Different samples of same production period. A. Column chromatography (*6*). B. Microbiological assay.

Table III. Amino Acid Composition of Yeast during Prolonged Propagation on Ammonia Spent Sulfite Liquor

(%, protein basis)

	Range	Average
Valine	6.2–6.5	6.3
Leucine	6.7–7.3	7.0
Isoleucine	4.6–5.7	5.3
Threonine	5.3–5.7	5.5
Methionine	1.0–1.3	1.2
Phenylalanine	4.2–4.6	4.3
Tryptophan	1.1–1.3	1.2
Histidine	1.8–1.9	1.9
Lysine	6.5–6.9	6.7
Glycine	4.7–5.1	4.8
Alanine	3.3–3.5	3.4
Serine	5.1–5.9	5.5
Cystine	0.4–1.0	0.7
Proline	3.5–3.6	3.5
Tyrosine	3.3–3.4	3.3
Aspartic acid	4.5–4.7	4.7
Glutamic acid	14.7–15.2	15.0
Arginine	5.3–5.5	5.4

Table IV. Folic Acid Content of Yeasts

	Samples	Range, μg./G.	Average, μg./G.
Saccharomyces cerevisiae (molasses grown)	'61 17	7.8–12.9	9.8
	'62 23	8.4–16.1	11.5
	'63 19	8.3–16.2	12.1
Candida utilis (sulfite liquor grown)	'61 14	7.9–12.9	9.1
	'62 23	9.2–17.1	12.7
	'63 17	9.4–18.1	12.6

Table V Free Folic Acid of Yeasts

	Range, mμg./G.	Average, mμg./G.
Saccharomyces cerevisiae	219–350	269
Candida utilis	730–964	821

After the switch from calcium base to ammonium base became permanent, its prolonged effect on the amino acid composition was studied. Samples in this period were taken at four points: at the start, and after 3, 13, and 25 weeks of discontinuous yeast production (Table III).

Vitamin samples were reserved independently of the amino acid studies. Daily composites of dried torula yeast N.F. XI (*Candida utilis*) and dried yeast N.F. XI (*Saccharomyces cerevisiae*) (*12*) were collected at random every 2 to 3 weeks over a 3-year period. The total folic acid content of 59 samples of *Saccharomyces* primary-grown yeast was compared with 54 samples of *Candida* food yeast (Table IV). Only 11 samples of each kind of nutritional yeast were spot-checked over a 2-year period for free folic acid content (Table V).

All assays for amino acids and vitamins were conducted by the laboratories of the Wisconsin Alumni Research Foundation, employing microbiological procedures, suitably modified (*2*, *3*, *8*).

Results and Discussion

Yeast performance on calcium and ammonium SSL over the 10-day test periods reflects reasonably good control for this type of substrate when one considers that a blend of hardwood and softwood, usually 1 to 1, was used in most of the cooks. The higher the proportion of hardwood in such mixtures, the greater the content of xylose and organic acids, and the longer the retention time of yeast in the fermentor; however, yeast yield (based on sugar) is enhanced by the additional conversion of the nonsugar carbon compounds. Preliminary oxygen-uptake measurements in the Warburg microrespirometer showed yeast activity on hardwood liquors from 70 to 75% of that on corresponding hexose-rich softwood liquors. Oxygen-uptake rates remain about the same when the hexose-pentose in hardwood-softwood mixtures is about 1 to 1 at equivalent sugar concentrations. These rates closely parallel those observed for pure sugar solutions and their mixtures.

Amino acid values obtained by microbial assay were higher than those by the Moore-Stein method (*5, 6*) for five of the nine amino acids considered essential for man (Table II). In general, good agreement is shown by the two methods, and neither assay procedure detected a change in amino acid composition of yeast grown on calcium SSL and then shifted to ammonium SSL substrates.

Similarly, prolonged propagation of *C. utilis* on ammonia-base liquor (Table III) produced food yeast of uniform amino acid composition. Again, the first nine compounds listed are those essential for man. The values for six of them are higher than those listed previously for the corresponding amino acids assayed chromatographically. In addition, the range of values for all amino acids is surprisingly narrow over the 6-month observation period.

While these assays have affirmed the high quality and uniformity of yeast protein following drastic changes in fermentation substrates, they also allow updating of product information (*1, 16*), and offer reliable data useful in nutritional projects elsewhere (*11, 13, 17*).

The fresh data on the folic acid content of food yeasts were obtained after the Food and Drug Administration removed dried yeasts from the GRAS list. Folic acid values (Table IV) accumulated for both types of dried yeast—*Candida utilis* and *Saccharomyces cerevisiae*—are essentially similar, but much lower than recorded by the earlier literature. Both kinds of yeast contain roughly only half as much folic acid as reported (*4, 9, 17*).

Values for free folic acid (Table V) reveal a consistently higher *Candida* food yeast content, about threefold greater than levels found in primary-grown *Saccharomyces* food yeast. Since the older data were inadequate (*4*), no meaningful conclusions can be drawn from comparisons with them.

Both kinds of yeast, however, contain folic acid levels well within the limits established by a recent Food Additives Order (*7*). This order provides that dried yeast (*C. utilis, S. cerevisiae*, or *S. fragilis*) may be safely used in food (as a flavoring), provided the total folic acid content of the yeast does not exceed 40 μg. per gram of yeast [approximately 8 μg. of free folic acid (pteroylglutamic acid) per gram of yeast]. In special dietary food usage, the limit is 400 μg. of folic acid per day (100 μg. of free folic acid per adult per day).

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Literature Cited

(1) Anderson, R. F., Jackson, R. W., *Appl. Microbiol.* **6,** 369 (1958).
(2) Assoc. Offic. Agr. Chemists, "Methods of Analysis," 8th ed., p. 830, 1955.
(3) *Ibid.*, 9th ed., p. 666, 1960.
(4) Doctor, V. M., Peterson, W. H., *Appl. Microbiol.* **3,** 29 (1955).
(5) Dubey, G. A., Gfeller, P., Wiley, A. J., Fermentation Subdivision, Division of Agricultural and Food Chemistry, 130th Meeting, ACS, Atlantic City, N. J., September 1956.
(6) Dubey, G. A., Wiley, A. J., Sulphite Pulp Manufacturer's Research League, Appleton, Wis., Internal Report, 1960.
(7) *Federal Register*, Part 121, Subpart D, 21CFR Section 125.1125 (May 17, 1963).
(8) Henderson, L. M., Snell, E. E., *J. Biol. Chem.* **172,** 15 (1948).
(9) Inskeep, G. C., Wiley, A. J., Holderby, J. M., Hughes, L. P., *Ind. Eng. Chem.* **43,** 1702 (1951).
(10) Kaiser, W., Jacobs, W., *Food Proc.*, **18,** No. 1, 22 (1957).
(11) Klapka, M. R., Dubey, G. A., Pavcek, P. L., *J. Am. Diet. Assoc.* **34,** 1317 (1958).
(12) National Formulary XI, American Pharmaceutical Association, Washington, D. C., 1960.
(13) *Pure Appl. Chem.* **7,** 147 (1963).
(14) *Tappi* **45,** 126A (1962).
(15) Wiley, A. J., in "Industrial Fermentations," L. A. Underkofler and R. J. Hickey, eds., Vol. I, 307, Chemical Publishing Co., New York, 1954.
(16) Wiley, A. J., Dubey, G. A., Lueck, B. F., Hughes, L. P., *Ind. Eng. Chem.* **42,** 1830 (1950).
(17) *Yeast* **1,** No. 5 (Anheuser-Busch, Inc., St. Louis, Mo.) (1951).

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NITRATE IN PLANT MATERIAL

Determination of Nitrate in Silages and Forages

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METHODS for the determination of nitrate in plant material have assumed increased interest with growing reports of nitrate toxicity in livestock (*1, 3, 5, 6, 8, 15*). The number and variety of available analytical methods, according to Morris and Gonzales-Mas (*10*), may be an indication that no single method is completely satisfactory.

Papenhagen (*12*) noted the limitations of colorimetric methods in respect to interferences by nitrites, chlorides, and organic and inorganic material. These criticisms applied wholly or in part to methods which involved nitration, oxidation, or reduction properties of the nitrate ion.

Methods which utilize the reduction of nitrate to ammonia require a separate analysis or removal of ammonia originally present. An error in either component analysis may give an amplified error in the estimate of nitrogen.

The color reaction of nitrates with ferrous sulfate is the basis of a method by Swann and Adams (*16*), which was modified by Morris and Gonzales-Mas