Production and Use of Microbial Enzymes for Food Processing

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Many commercial enzymes are derived from strains of *Aspergillus flavus-oryzae*, *Aspergillus niger*, and *Bacillus subtilis*, which have had a long history of safe use in food processing. These commercial enzymes are produced by submerged culture and semisolid culture procedures. The important steps of organism selection, culture maintenance, plant fermentations, extraction, clarification, evaporation, use of preservatives, precipitation, drying and grinding, control of quality and uniformity, and formulation are discussed.

Microbial enzyme preparations have been widely used for a variety of purposes in the production of numerous food products for a great many years. Their practical application in fermented products dates back many centuries, long before the nature and function of enzymes, or even the microorganisms themselves, were known or understood. All fermentation processes, including those for fermented food products, are manifestations of enzyme reactions. Numerous types of organisms are employed in the preparation of fermented food products, such as yeasts in bread and other raised baked goods, beer and wine, bacteria of various types and species in pickles, sauerkraut, olives, and vinegar, various bacteria and molds in cheeses, and molds in soy sauce.

After the nature of enzymes became known, it was natural that they should be isolated from the organisms employed in fermentation processes, because the isolated enzymes act more specifically, their potency can be better standardized, and they are easier to handle and to store than the intact organisms. Such isolated microbial enzymes have had a long history of use in commercially preparing food and feed products such as bread, crackers, corn sirups, meat products, precooked cereals, fruit and fruit juices, wines, beers, vinegars, cheeses, egg solids, candies, chocolate sirups, liquid coffee concentrates, condensed fish solubles, and distillers' by-product feeds (*4*, *7–9*, *13–15*, *17–19*, *23–26*, *28*, *29*).

The function of enzymes in the production of foods varies widely. In some processes the enzyme product is used to accomplish a result more efficiently and better than other agents. In other processes the enzyme products are used because no other method produces the desired result. In most cases the added enzyme remains in the finished food. The enzyme activity, however, may be destroyed, as most food products in which enzymes are used are heated during processing to a temperature sufficient to inactivate the enzyme. The concentration of enzyme preparation used varies greatly, depending upon such factors as pH, temperature, and reaction time allowed. When calculated on the basis of the amount of material employed, the usage rate for most applications is in the order of 0.001 to 0.1% of the enzyme preparation in the food on the basis of solids.

Most of the commercially available microbial enzymes useful in food processing have been derived from strains of yellow-green Aspergilli of the *Aspergillus flavus-oryzae* group, of the black Aspergilli of the *Aspergillus niger* group, and of *Bacillus subtilis*. This is probably because of the ubiquitous nature of these organisms and the wide variety of enzymes produced in high yields by the different strains. The enzyme systems produced by these organisms are designated: Oryzae enzymes, Niger enzymes, and Subtilis enzymes.

However, many other types of nonpathogenic and nontoxic organisms could well be employed as sources for useful enzymes. Some examples are yeast strains for invertase or lactase, Rhizopus strains for amylase or glucamylase, and Penicillium strains for pectinase or lipase. Enzyme products from some of these sources have a limited market at the present time.

In Table I are tabulated representative commercial applications of microbial enzymes in foods in the United States. This is not intended to be a complete listing, since there are other microbial enzyme products derived from the same or other microbial sources, and other applications for the enzyme products.

Enzyme Production

A knowledge of the methods used in producing commercial microbial enzymes is essential to an understanding of the character of the products. Certain phases of the commercial production of enzymes have been described in patents and scientific publications (*4*, *8*, *14*, *15*, *17–19*, *23*, *24*, *29*). The exact steps of procedure vary somewhat among different manufacturers, but the methods of cultivation of the organisms may be classified as either the liquid submerged culture method or the semisolid medium method. The flow diagram of Figure 1 outlines the two procedures.

Selection of Organisms. The first step in the manufacture of any commercial microbial enzyme is the selection of an organism that, when grown in pure culture, produces the desired enzyme in good yield. In this paper we are concerned with the enzyme products derived from the bacterium *Bacillus subtilis*, and fungi from the *Aspergillus flavus-oryzae* and *Aspergillus niger* groups.

The specific strains of the organisms employed in enzyme production were originally obtained by isolation from natural sources or from cultures maintained in established culture collections such as those of the American Type Culture Collection, the Northern Utilization Research Branch, or other governmental or academic institutions. Rigid maintenance of cultural purity and freedom from any variation whatever are essential for their use in enzyme production.

Subtilis enzymes are produced by strains of *Bacillus subtilis*. Various strains of this widely distributed, nonpathogenic, aerobic, catalase-positive organism may differ slightly in minor characteristics but fit the characterization of "Bergey's Manual of Determinative Bacteriology" (*5*).

Oryzae enzymes are produced by members of the *Aspergillus flavus-oryzae* group, and Niger enzymes are produced by members of the *Aspergillus niger* group. The strains used conform to the general characteristics ascribed to these groups according to the classification of Thom and Raper (*20*).

Culture Maintenance. All strains are maintained as pure cultures by using accepted microbiological techniques.

These methods include cultivation on agar slants and storage as soil cultures or lyophilized cultures. Purity of the cultures is assured by using methods to exclude contaminating organisms and to avoid development of substrains from within the culture itself, and by conducting regular microscopic and plating tests. A competent microbiologist has no difficulty in recognizing the organisms in their cultures by observation of macroscopic colony characteristics and microscopic morphology. As an additional precaution in routine manufacturing use, single spore or cell isolates are made at frequent intervals and are tested for morphological and biochemical characteristics to assure purity of strain and maximum enzyme production. By use of these techniques, purity of culture and purity of strain are maintained indefinitely, and in this way cultures of uniform characteristics are supplied for enzyme production.

Preparation of Inoculum. As each culture is prepared for use as inoculum for production, it is checked for purity and freedom from variant forms, for physiological characteristics, and for enzyme formation.

For inoculation of large commercial batches of nutrient media, large numbers of inoculating organisms are required. These inocula are prepared under rigid standards of aseptic technique. The laboratory pure culture is used to inoculate either flasks of sterile liquid nutrient medium or bottles of sterile moistened semisolid medium. The various media employed consist of the same ingredients used in the production mashes. The flask or bottle inoculum cultures are grown in laboratory incubators under controlled temperature conditions. After checking for purity, they may be used directly for inoculation of production medium, or built up in volume through seed tanks of intermediate size containing production media. Operating and control procedures similar to those used in production fermentations are applied to seed tanks. The criteria for optimum growth in seed tanks are established to give a culture of the greatest vitality. In general, this involves a combination of the number of cells and the physiological state of the cells. Growth is followed by periodic examination of samples. Growth in normal seed tank will follow a predicted pattern with respect to such criteria as microscopic cell appearance, rate of cell growth, disappearance of medium ingredients, and pH change. Any deviation from the normal pattern is a basis for rejection of that batch of inoculum. All inoculum and seed tank cultures are carefully checked for purity and typical growth and development before they are used.

Plant Fermentation. In Table II are listed various nutrient ingredients, combinations of which are used to produce the commercial microbial enzymes. The exact compositions of the media employed will vary with the manufacturer, the strain of organism, the enzyme system desired, and the type of growing procedure used. Every effort is made to adhere strictly to the optimum medium formulations and fermentation conditions for the different enzyme products. Strict control of medium composition is vital to consistently high and uniform enzyme yields.

Table I. Representative Microbial Enzyme Applications

Common Names	Major Enzyme Type	Typical Uses	Maximum Levels in Food, P.P.M.[a]	In Common Use Since
Subtilis enzymes	Carbohydrase	Chocolate sirup (viscosity control)	100	1929
		Brewing (liquefaction of cooker mash in brewing and distilling)	100	1936
		Precooked cereals (modification of cereal starches to improve characteristics)	500	1952
	Protease	Brewing (to maintain clarity of beer)	10	1940
		Protein hydrolyzates	500	1947
		Crackers (modification of dough)	40	1952
		Condensed fish solubles for animal feed (viscosity control)	1000	1950
Oryzae enzymes	Carbohydrase	Production of high conversion sirups from acid-hydrolyzed starch (corn sirup)	250	1940
		Saccharification of distiller's mash (alcohol production)		
		In alcohol product	0	1943
		In distiller's grain (feed)	1000	1943
	Carbohydrase	Fruit juices (clarification)	400	1922
		Brewing (removal of starch from wort)	10	1931
		Chocolate sirup (viscosity control)	200	1931
	(Amylase) (Protease)	Bread and cracker baking (modification of dough)	50	1918
	Protease	Meat tenderizer	500	1950
Niger enzymes	Carbohydrase	Saccharification of distillers' mash (alcohol production)		
		In alcohol product	0	1943
		In distiller's grain (feed)	1000	1943
	Cellulase	Liquid coffee concentrate (viscosity control)	100	1952
	Glucose oxidase-catalase	Egg solids production (removal of glucose)	750	1952
		Beverage and food products (removal of oxygen)	10	1955
	Pectinase(s)	Fruit juice and wine (production and clarification)	200	1930
	Lipase	Cheese (flavor production)	100	1952

[a] Enzyme concentrate (usually inactivated) excluding diluents.

Submerged Culture. Enzyme fermentations by the submerged culture method are carried out in closed fermentors equipped with agitators, aeration devices for introduction of sterile air, and jackets or coils for temperature control. The fermentors are cleaned with caustic alkali and/or detergent solutions and water, and are sterilized with live steam between batches.

The liquid media can be sterilized on a continuous basis, the sterilized media going into sterile fermentors, or the ingredients can be introduced into the fermentor and the medium sterilized in the fermentor by heating. The sterile mash in the fermentor is cooled to 25° to 40° C. under pressure, using air sterilized by passage through sterile filters of activated carbon, glass wool, or similar type of filtering agent. The pH may be adjusted after sterilization with acid or alkali if required. Sterility of each batch of medium is checked by standard bacteriological techniques.

The cooled medium in the fermentor is inoculated with pure inoculum culture of the organism. Suitable agitation and aeration are supplied to the growing culture, and the temperature is maintained by circulating water through the coils or jackets of the fermentor. Samples are taken periodically during the fermentation for control testing. Continuous controls of temperature, pH, disappearance of certain mash ingredients, purity of culture, and level of enzyme are exercised. Because of strict control, and the uniformity of culture, mash composition, and operating procedures, each fermentation follows a well defined pattern. Optimum harvest time

is the point at which the laboratory tests show that maximum production of enzyme activity has been achieved. The fermentation period may be from 24 to 120 hours, depending upon the enzyme system being produced.

Semisolid Culture. Enzyme production by the method of culture on semisolid media is carried out in large chambers fitted with trays or in horizontal rotating drums. The chambers, trays, and drums are washed with detergent, sanitized with sodium hypochlorite or formaldehyde, and steamed between successive batches. The nutrients, consisting of wheat bran and other ingredients listed in Table II, are mixed with water and subjected to flowing steam or steam under pressure for 30 to 120 minutes. The hot mass is then cooled to 25° to 40° C. and inoculated with pure inoculum culture.

When horizontal revolving drums are used, the mash is inoculated in the drum. Temperature is controlled by water flowing over the drum and the passage of filtered air through the drum. The drum revolves slowly during the growth period.

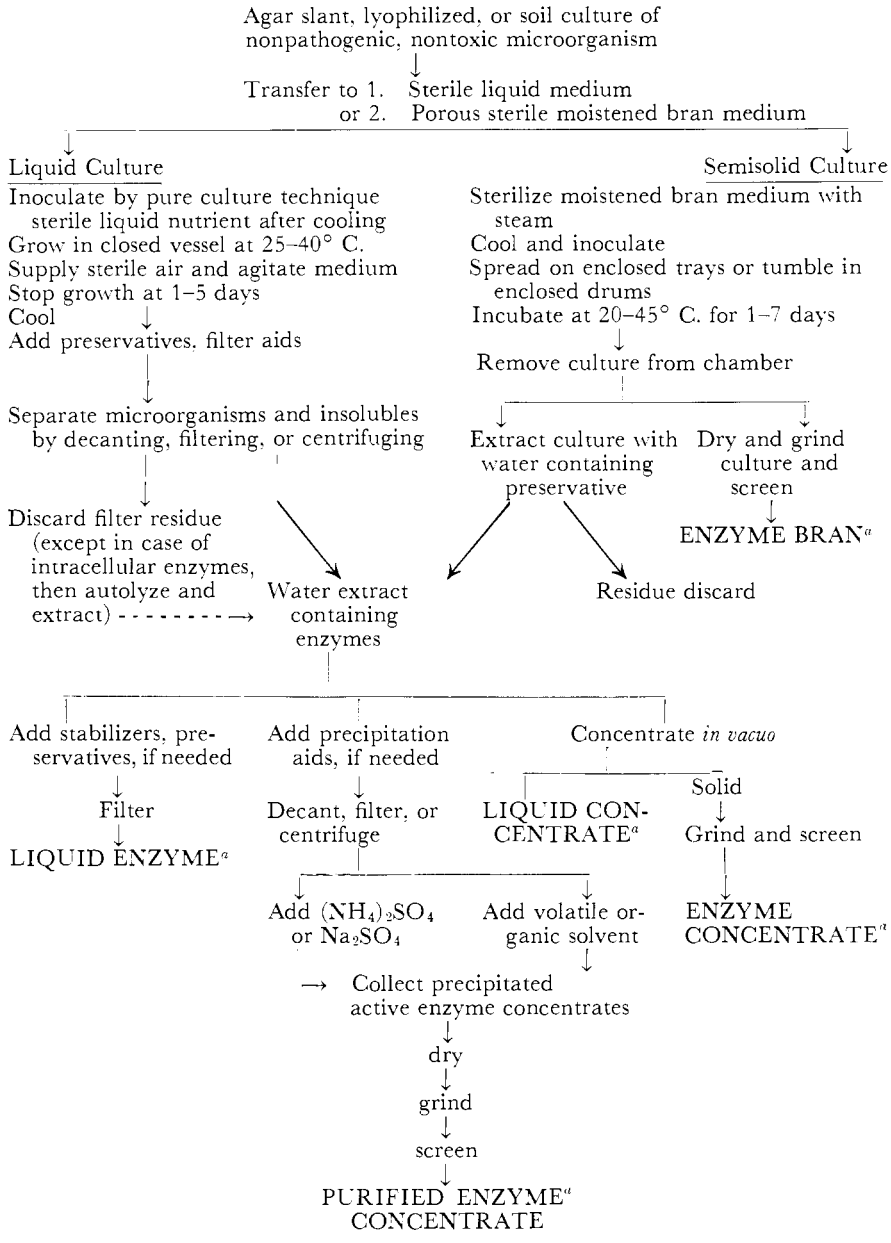
When trays are used, the inoculated material is conveyed to the chamber in carts, steamed before use, then spread in layers on the clean trays with perforated bottoms. These are placed on racks and incubated in the chambers until maximum enzyme production has occurred. The time may vary from 1 to 7 days with different organism strains. The temperature of the growing chamber is maintained by circulating cool, humidified air through the chamber, or by passing water through the interior of the trays supporting the culture. The porous structure of the bran medium allows air to penetrate throughout.

During growth in drums or trays the temperature, pH, moisture level, purity of culture, and enzyme level are carefully determined on composite samples and controlled where applicable. During growth a significant loss in weight takes place due to oxidation of organic material to carbon dioxide and water. Optimum harvest time is the point where laboratory tests indicate that maximum production of enzyme activity has been attained.

Extraction. Where liquid mash is employed, the desired enzymes are usually extracellular and are present in the free liquor; hence no extraction step is required. In the case of enzymes retained within the cells, the cells are removed by filtration. The liquor is discarded and the filter cake is slurried in water containing calcium chloride and/or phosphates. The cells are autolyzed, and the cell debris is removed by filtration or centrifugation, yielding a clear extract for further processing.

For semisolid mashes, extraction of the enzyme may be performed on the moist material directly upon completion of growth, or the culture mass may be dried and subsequently extracted. The mass may be extracted with water which may contain a preservative. A common procedure is to use a countercurrent extraction system that filters as well as extracts and gives an extract containing soluble material that is relatively free from both the organism and insoluble particles from the medium.

Figure 1. Flowsheet of microbial enzyme manufacture



Both liquid culture and semisolid culture procedures can be and are used for the production of all the types of enzyme products covered in this review.

[a] These products can be diluted to standard activity.

Clarification. Fermentation beers from liquid mash culturing and extracts from semisolid mash culturing contain the enzymes in solution, along with suspended solids including materials from the media and microbial cells or mycelium. One or more of the materials listed in Table III are added to the liquor for special purposes such as stabilization or activation, adjusting the pH, flocculation of the liquor, or improving filtration rate. These are generally used at a low level approximating 1% or less. After addition of the agents the extract is clarified by filtration or centrifugation.

Evaporation. The clarified filtrate may be used for precipitation directly, or it may be concentrated severalfold prior to precipitation, or for formulation into liquid enzyme products. When concentration is required, it is effected in a low temperature vacuum evaporator.

Preservatives. At certain stages during the purification process, small amounts of preservatives may be used to inhibit growth and contamination by foreign organisms. Those used, when necessary, include toluene, organic acids or their salts, phenolic compounds, and sodium fluoride. Some of the preservatives used are entirely transient—that is, they leave no residue in the final product. However, others may leave residues in the enzyme concentrates not exceeding

Table II. Production Media Ingredients

Subtilis Enzymes		*Oryzae Enzymes*		*Niger Enzymes*	
Liquid culture	*Semisolid culture*	*Liquid culture*	*Semisolid culture*	*Liquid culture*	*Semisolid culture*
Cereal starch or starch hydrolyzate	Wheat bran	Cereal starch or starch hydrolyzate	Wheat bran	Cereal starch or starch hydrolyzate	Wheat bran
Milled cereal products such as grits, meal, flour	Milled cereal products such as middlings, second clears, flour	Milled cereal products such as grits, meal, flour	Milled cereal products, such as middlings, second clears, flour	Milled cereal products such as grits, meal, flour	Milled cereal products such as middlings, second clears, flour
Corn steep liquor	Soybean meal	Corn steep liquor	Soybean meal	Beet pulp, citrus pulp, or apple pomace	Soybean meal
Soybean meal	Corn steep liquor	Soybean meal	Corn steep liquor	Corn steep liquor	Beet pulp, citrus pulp, apple pomace
Distillers' solubles	Yeast extract	Distillers' solubles	Yeast extract	Yeast or yeast extract	Corn steep liquor
Yeast or yeast extract	Peptone	Yeast or yeast extract	Peptone	Casein	Yeast extract
Casein	Distillers' solubles	Casein	Lactic or hydrochloric acid	Peptone	Peptone
Gelatin	Ammonium hydroxide	Gelatin	Distillers' solubles	Dextrose	Lactic or hydrochloric acid
Peptone	Ammonium phosphate	Peptone	Ammonium hydroxide	Tannic acid	Ammonium phosphate
Lactose	Dipotassium phosphate	Lactose	Ammonium phosphate	Ammonium phosphate	Ammonium sulfate
Dextrose	Monopotassium phosphate	Dextrose	Dipotassium phosphate	Calcium carbonate	Urea
Tannic acid		Tannic acid	Monopotassium phosphate	Sodium chloride	
Ammonium phosphate		Ammonium phosphate	Urea	Magnesium sulfate	
Calcium carbonate		Calcium carbonate		Dipotassium phosphate	
Sodium chloride		Sodium chloride		Monopotassium phosphate	
Magnesium sulfate		Magnesium sulfate		Ammonium hydroxide	
Manganous chloride		Manganous chloride		Sodium hydroxide	
Dipotassium phosphate		Monopotassium phosphate			
Monopotassium phosphate		Dipotassium phosphate			
Disodium salt of ethylene (dinitrilo)tetraacetic acid		Alkyl aryl polyether alcohol (antifoam)			
Ammonium hydroxide		Iron ammonium citrate			
Sodium hydroxide		Ammonium hydroxide			
		Sodium hydroxide			

10 p.p.m. At the normal enzyme levels used in food processing, final levels in foods would never exceed 0.05 p.p.m.

Precipitation. The enzyme is precipitated by adding one or more of the precipitants and coprecipitants listed in Table IV to the filtrate or liquid concentrate. Precise controls of the quantity of ingredient, mode of addition, and temperature are required. Following precipitation, the material is filtered or centrifuged, using conventional techniques. The cake of enzyme precipitate may be sparged with materials from the list in Table IV.

Drying and Grinding. The enzyme cake is dried in atmospheric or vacuum dryers at low temperature. After all traces of solvent have been removed, it is ground to a suitable particle size and used in the preparation of enzyme formulations.

Characteristics of Enzyme Products

The active ingredients in the commercial enzyme concentrates produced by the foregoing processes are, of course, the enzymes themselves. Present also are certain components remaining from the original nutrients and produced as metabolic products by the growing organism, all of which are normally present in various food products. The proximate analysis of a typical alcohol-precipitated microbial enzyme preparation shows:

Moisture, %	5–7
Protein, %	30–40
Carbohydrate, %	35–40
Ash, %	10–20
Ether-extractable, %	0.01–0.1

Table III. Agents Used in Clarification of Enzyme Extracts

Ammonium phosphate, dibasic	Gelatin
Ammonium phosphate, monobasic	Gum arabic
Ascorbic acid	Hydrated lime
Calcium salts (chloride, formate, sulfate, or phosphate)	Hydrochloric acid
Cellulose fiber	Phosphoric acid
Cysteine	Sodium citrate
Diatomaceous earth	Sodium gluconate
	Sodium phosphate, monobasic
	Sodium sulfite

Table IV. Agents Used in Precipitation of Enzymes

Acetone[a]	Gelatin
Ammonium sulfate	2-Propanol[a]
Casein	Lactose
Diatomaceous earth	Methanol[a]
Disodium phosphate	Sodium sulfate
Ethyl acetate[a]	Starch
Ethyl alcohol[a]	

[a] Transient.

Because commercial microbial enzyme products are derived by fermentations in which only edible materials are used as nutrients and only nonpathogenic organisms are employed which do not produce harmful metabolic end products (*5*, *20*), they are inherently safe. This is further substantiated by the many years of their safe use.

Control of Quality and Uniformity. To ensure the quality of enzyme products, high standards of cleanliness are maintained in the place of manufacture. The standards enforced are fully equal to those found in the best food plants. Control is maintained by extensive laboratory supervision of all phases of the process together with good housekeeping and refrigeration.

Enzyme products are standardized with respect to potency by means of assay procedures based on specific types of functional activity—that is, enzyme assay procedures are based upon the amount of substrate changed by the enzyme under rigidly controlled conditions. Assay procedures are employed which are well known in the enzyme literature, sometimes modified slightly for convenience in routine use or to improve accuracy and reproducibility (*1–3*, *6*, *10–12*, *16*, *21*, *22*, *27*).

Formulation. Commercial enzyme products are offered in three forms: enzyme-bran, liquid, and precipitated purified powders.

The enzyme-bran products are similar to wheat middlings in texture and appearance. They are usually standardized with respect to functional enzyme activity for a particular purpose. The diluents or standardizing agents used include wheat middlings, bran, salt, and wood flour. The insoluble diluents may be removed by filtration in the processes in which the enzymes are employed.

The liquid products range in color and appearance from those that may

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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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