[CONTRIBUTION FROM THE LABORATORY OF CHEMISTRY AND CHEMOTHERAPY, EXPERIMENTAL BIOLOGY AND MEDICINE INSTITUTE, NATIONAL INSTITUTES OF HEALTH]

The Preparation of Trehalose from Yeast[1]

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Although the disaccharide trehalose has been known for more than one hundred years,[2] its isolation from such a convenient source as baker's yeast was not accomplished until 1925. In that year Koch and Koch,[3] in connection with their studies on "bios," permitted an alcohol extract of air-dried Fleischmann's compressed yeast to stand undisturbed for several months; at the end of that time they found a cluster of well-formed crystals clinging to the side of the flask. In a subsequent, large-scale extraction they obtained sufficient material to purify by several recrystallizations and to identify conclusively as trehalose. By a very similar extraction procedure Kluyver and van Roosmalen[4] were able to prepare 90 g. of trehalose (0.3% yield) from 27 kg. of dried Delft compressed yeast.

Tanret[5] introduced the use of basic lead acetate as a deproteinizing agent, and reported yields of 20 g. of trehalose per kilogram of vacuum-dried baker's yeast and 5.5 g. per kilogram of the fresh yeast containing 28% solids. However, the yield of trehalose from the same yeast after being dried for eight days in the air at 25° or less was only 7.5 g. per kilogram on a dry basis.

Steiner and Cori[6] isolated trehalose from baker's yeast by extraction with *N* sulfuric acid,[7] followed by a precipitation with mercuric and ferric sulfates in sulfuric acid, neutralization with barium carbonate, filtration and subsequent removal of heavy metals with hydrogen sulfide. Concentration of the neutral aqueous solution yielded about 2 g. of crystalline trehalose from 300 g. of compressed yeast. From hydrolysis experiments with the deproteinized extracts Steiner and Cori reported that fresh baker's yeast contains from 0.5 to 1.5 g. of trehalose per 100 g. moist weight, and that during fermentation of glucose the trehalose content of the yeast may increase to 2 to 3%.

Myrbäck and Örtenblad[8] have described a combination of the above methods, namely, extraction of the baker's yeast with alcohol, concentration to remove most of the solvent, and deproteinization of the aqueous solution by precipitation with mercuric sulfate in sulfuric acid and subsequent neutralization with barium carbonate. The filtrate, freed from mercury with hydrogen sulfide, was then concentrated and the trehalose crystallized by the addition of alcohol. The yields reported from Swedish compressed yeast were up to 25 g. of trehalose dihydrate per kilogram, or up to 10% of the dry weight.

In order to obtain large amounts of trehalose from baker's yeast we have found it desirable to modify the procedure in two ways. The compressed yeast was extracted with alcohol and the solvent removed by concentration as before. The aqueous solution was deproteinized by an adaptation of Somogyi's method for blood,[9] that is, by the addition of zinc sulfate followed by enough barium hydroxide to make the mixture pink to phenolphthalein. The filtered solution was freed from all ionizable material including amino acids by passage through a suitable pair of ion-exchange columns, then concentrated and the trehalose crystallized in practically pure condition by the addition of alcohol.

By the above modification it was possible to isolate readily nearly all the trehalose that was present in four different commercially available baker's yeasts (see Table I). However, the actual trehalose content, as has been shown by the work of Tanret,[5] Steiner and Cori,[6] Myrbäck and Örtenblad,[8] and Brandt,[10] varies with the history and particularly with the age of the yeast. In confirmation of these previous reports, we found the highest trehalose content of one brand of compressed yeast to be 20 g. per kilogram; other samples, obtained at two-week intervals directly from the press and analyzed immediately, showed 17.3, 13.1 and 9.2 g. per kilogram, respectively. Three other brands of compressed yeast contained trehalose in varying amounts which were more or less inversely proportional to the length of time the product had been in transit. In the laboratory at 20° the fresh yeast usually lost 90–100% of its trehalose content within six days, while at refrigerator temperature (+5°) about six weeks was required before a corresponding loss occurred (see Table II). Although drying has been suggested as a means of preserving the trehalose content of compressed yeast, the laboratory preparation of dried yeast is troublesome and may involve a considerable loss of the sugar. We have now found that up to 100% of the original

(1) A preliminary report of this research appeared in THIS JOURNAL, **71**, 2277 (1949).

(2) See, for example, "Beilsteins Handbuch der organischen Chemie," Fourth Edition, Verlag von Julius Springer, Berlin, 1938, Vol. 31, p. 378.

(3) E. M. Koch and F. C. Koch, *Science*, **61**, 570 (1925).

(4) A. J. Kluyver and F. L. W. van Roosmalen, *Biochem. Z.*, **245**, 13 (1932).

(5) G. Tanret, *Compt. rend.*, **192**, 1056 (1931); *Bull. soc. chim. biol.*, **13**, 598 (1931).

(6) A. Steiner and C. F. Cori, *Science*, **82**, 422 (1935).

(7) Dr. Cori has recently informed us in a personal communication that through a typographical error the expression *N* H_2SO_4 appeared as NH_2SO_4, and this in turn in *C. A.*, **30**, 566 (1936), became $(NH_4)_2SO_4$.

(8) K. Myrbäck and B. Örtenblad, *Biochem. Z.*, **288**, 329 (1936); K. Myrbäck, *Svensk Kem. Tid.*, **48**, 55 (1936).

(9) M. Somogyi, *J. Biol. Chem.*, **160**, 69 (1945).

(10) K. M. Brandt, *Biochem. Z.*, **309**, 190 (1941).

trehalose content can be preserved for a year or more at room temperature simply by covering the compressed yeast with alcohol as soon as it is received. Since this is normally the first step in the isolation of trehalose, this procedure enables an investigator to postpone the succeeding steps, without danger of losing the trehalose, until he has further opportunity to complete his work.

A paper by Payen[11] which appeared just as our paper was being written stated that in the drying process used in the preparation of live dried baker's yeast the glycogen content decreased and the trehalose content increased, so that the final product contained as much as 18% trehalose. We have been able to confirm that active dried yeast[12] is indeed a rich source of trehalose; a 320-g. sample of that yeast, containing only 8% moisture, when processed by the procedure described here, yielded readily 52 g. of crystalline trehalose (16%).[12a]

Although Müntz[13] did not examine baker's yeast in connection with his studies on trehalose and mannitol in mushrooms, he did report that he could find no trehalose in brewer's yeast. Tanret,[5] Myrbäck and Örtenblad,[8] and more recently Bouthilet, Neilson, Mrak and Phaff[14] also have reported that they were unable to detect the presence of any trehalose in brewer's yeast. However, we have succeeded in isolating crystalline trehalose readily and in yields up to 12 g. per kilogram from four fresh waste beer yeasts and two ale yeasts from several different breweries (see Table I). These results are not too surprising in view of the known formation of trehalose monophosphate through the phosphorylation of glucose by brewer's bottom yeast in the presence of toluene,[15] and the very recently reported isolation of crystalline trehalose after fermentation of glucose by maceration juice prepared from brewer's yeast.[16] The reasons for the failure of previous investigators to isolate or even to obtain any evidence of the presence of trehalose in brewer's yeast are not clear from their papers, but our results show that fresh waste brewer's yeast must now be considered as a cheap and readily available source of trehalose.

On the other hand, we were unsuccessful in two attempts to detect trehalose in two samples of a distiller's yeast that was growing actively on a mash consisting of 50% rye and 50% barley malt. This cannot be construed as evidence that distiller's yeast never contains trehalose, but only that under the conditions of active growth on that particular medium the yeast did not store trehalose to be used as a reserve carbohydrate.[17]

Experimental Part

Isolation of Trehalose from Compressed Baker's Yeast.—Each kilogram of compressed yeast,[18] containing about 30% solids, was crumbled into a beaker and extracted with 2.5 liters of 95% alcohol by allowing it to stand, with occasional stirring, for about thirty minutes. The mixture was filtered on a large Büchner funnel and the filter cake washed with three 300-ml. portions of 70% alcohol. Further extraction of the yeast was found to be unnecessary and yielded only a negligible amount of trehalose. The combined filtrates were concentrated *in vacuo* to about 300 ml. to remove the alcohol. To the residual aqueous solution was then added 200 ml. of 20% zinc sulfate solution followed by enough saturated aqueous barium hydroxide solution (about 500 ml.) to make the mixture pink to phenolphthalein. Twenty grams of activated carbon was added, the mixture heated to about 70° in a boiling water-bath, and filtered through a Büchner funnel precoated with Filter-Cel. The rotation of the clear, colorless filtrate, calculated in terms of trehalose dihydrate, was found to be in good agreement with the actual weight of the crystalline sugar that could be isolated later.

To remove ionizable material the solution was passed through two columns of suitable ion-exchange resins. We used Amberlite IR-100 and Amberlite IR-4B, the active columns being about 55 cm. long and 5 cm. in diameter. Any amino acids originally present were also removed, and a negative ninhydrin reaction, indicating their absence, was used as evidence of complete deionization before a conductivity meter became available. The rotation of the deionized solution usually showed a small increase calculated as trehalose dihydrate; this was presumably due to the removal of other optically active substances, including amino acids, whose net rotation was slightly negative.

The deproteinized and deionized solution was concentrated *in vacuo* to a sirup which was transferred to a 500-ml. Erlenmeyer flask, and in a final volume of 50 ml. was diluted slowly with 400 ml. of 95% alcohol; if the estimated amount of trehalose is small, these volumes may be decreased. The trehalose usually began to crystallize spontaneously, and after a day at room temperature and several days in the refrigerator it was filtered and washed with alcohol. The trehalose dihydrate, in large transparent prisms, was practically pure and represented up to 95% of the sugar present as calculated from the earlier rotation values. Additional amounts could be obtained from the mother liquor although they were sometimes contaminated by the deposition on them of fine whitish prisms of *meso*-inositol. No easy method was found for the separation of these two substances on a small scale, but additional trehalose could be recovered by combining a

(11) R. Payen, *Can. J. Research*, **27B**, 749 (1949).

(12) "Fleischmann's Fast Rising Dry Yeast," an active dried yeast, was furnished through the courtesy of the Fleischmann Manufacturing Division of Standard Brands, Incorporated, 595 Madison Avenue, New York, N. Y.

(12a) *Note added December 30, 1949.*—The trehalose content of this type of yeast does not appear to diminish when it is kept under refrigerated storage. Mr. George W. Kirby kindly sent us samples 5F1075 (October 1945) and 7F1447 (November 1947); the yields of crystalline trehalose from 320-g. portions of these yeasts containing 8% moisture were 57.4 g. (18%) and 53.2 g. (17%), respectively. If 95% alcohol is not readily available for extracting the trehalose from the yeast, equally good results may be obtained by using a denatured alcohol of the type ten volumes of ethyl alcohol to one volume of methyl alcohol. Incidentally, the yeast cake left after removal of the trehalose can be utilized further for the production of nucleic acid [see, for example, G. Clarke and S. B. Schryver, *Biochem. J.*, **11**, 319 (1917)].

(13) A. Müntz, *Compt. rend.*, **79**, 1182 (1874); *Ann. chim. phys.*, [5] **8**, 56 (1876).

(14) R. J. Bouthilet, N. E. Neilson, E. M. Mrak and H. J. Phaff, *J. Gen. Microbiol.*, **3**, 282 (1949).

(15) S. Veibel, *Biochem. Z.*, **239**, 350 (1931).

(16) M. Elander and K. Myrbäck, *Arch. Biochem.*, **21**, 249 (1949); see also H. Sobotka and M. Holzman, *Enzymologia*, **1**, 168 (1936), and R. Nilsson and F. Alm, *Acta Chem. Scand.*, **3**, 213 (1949).

(17) See especially Brandt, ref. 10.

(18) Compressed baker's yeast was purchased from Standard Brands, Inc., Langdon, D. C., Federal Yeast Corporation, Baltimore, Md., and Red Star Yeast and Products Company, Milwaukee, Wis. A fourth sample was furnished through the courtesy of Dr. G. S. Bratton, Anheuser-Busch, Inc., St. Louis, Mo.

number of smaller batches and crystallizing the mixture fractionally from water by the addition of alcohol.

Isolation of Trehalose from Active Dried Baker's Yeast.—A 320-g. sample of this yeast,[12] containing 92% solids as determined by drying to constant weight at 105°, was made into a paste with 680 ml. of water, and was thus equivalent to 1 kg. of the compressed yeast of 29% solid content. The product was extracted, deproteinized and deionized as described above, concentrated to a volume of 100 ml. and diluted slowly with 800 ml. of 95% alcohol. The trehalose was allowed to crystallize for one day at 20° and two days in the refrigerator, then filtered, washed and dried; the yield was 52 g. of trehalose dihydrate of $[\alpha]^{20}D$ +179° in water (c, 2).

Isolation of Trehalose from Brewer's Yeast.—The samples of fresh waste yeast from the several breweries[19] were brought to the laboratory in cold, gallon thermos jugs, filtered in a room at 5° on large Büchner funnels, washed with water and pressed dry with the aid of a sheet of dental rubber dam material fastened over the top of the funnel with large rubber bands. Filtration of the bottom (beer) yeasts was completed overnight, but the slimier top (ale) yeasts usually required one or more days to reach the same solid content of 27–29%. These brewer's yeasts were then extracted, deproteinized, deionized, and the product obtained in crystalline form by the same procedure that was described for the baker's yeasts. A complete identification as trehalose from both brewer's bottom and top yeasts was established through rotations, melting points and mixed melting points.

Attempted Isolation of Trehalose from Distiller's Yeast.[20]—Two samples of this yeast were made available for our studies. The first was growing actively on a strained sour mash; it was separated and washed by centrifugation to yield 485 g. of yeast containing 15% solids. The rotation of the deproteinized alcohol extract corresponded to 0.17 g. of trehalose. The second sample was growing on the mash itself consisting of equal amounts of rye and barley malt; the mixture was filtered through cheesecloth, the filtrate centrifuged to separate the yeast from the finer particles of the mash, and the yeast then washed and centrifuged again to yield 280 g. of 20% solid content. The rotation of its deproteinized alcohol extract corresponded to 0.1 g. of trehalose. The solutions from the two batches were combined, deionized and concentrated, but no trace of trehalose could be induced to crystallize.

Determination of Trehalose Content in Small Samples of Yeast.—In view of the fact that the observed rotations of the deproteinized alcohol extracts of both baker's and brewer's yeasts corresponded very closely to the actual amount of trehalose that could be isolated later, the same method may be adapted to determine rapidly and fairly accurately the trehalose content of small samples of yeast. The high specific rotation of trehalose dihydrate (+178°) enables one to obtain satisfactory results from 10-g. samples and even from 1-g. samples which are rich in trehalose. The difficulties encountered in getting uniform samples and then weighing them when the moisture content is as high as 70–80% can easily account for the small variations obtained in duplicate analyses or from the values obtained with kilogram lots of the same yeast.

(19) Beer yeast was furnished through the courtesy of Mr. Francis R. Omlor of the Christian Heurich Brewing Co., Washington, D. C., and Mr. R. J. Klussmann of the American Brewery, Inc., Mr. F. X. Schneider of the Gunther Brewing Co., Inc., and Mr. C. R. Kreitler of the National Brewing Co., all of Baltimore, Md. Ale yeast was furnished through the courtesy of Mr. L. S. Cohen of the Gunther Brewing Co., Inc., and Mr. W. H. Ruppert of the Free State Brewery Corporation, both of Baltimore, Md., and Mr. F. E. Connery of P. Ballantine and Sons, Newark, N. J.

(20) Furnished through the courtesy of Mr. Philip Hickman of the Calvert Distillery, Relay, Md.

TABLE I

SOME REPRESENTATIVE DATA ON TREHALOSE IN YEAST

Source	History	Trehalose in g./kg. From rotation	Crystallized
Baker's 1	Fresh (29%)	18.3	16.0[a]
Baker's 1	Fresh (29%)	13.4	12.7[a]
Baker's 2	Fresh (29%)	17.0	
Baker's 3	Fresh (29%)	9.6	
Baker's 4	Fresh (29%)	3.9	
Baker's 5	Active dried (92%)	168	163[a]
Brewer's beer 1	Fresh (27%)	10.4	9.7
Brewer's beer 2	Fresh (27%)	4.3	2.7[a]
Brewer's beer 3	Fresh (26%)	8.8	7.5[a]
Brewer's beer 4	Fresh (26%)	4.5	3.4[a]
Brewer's ale 1	Fresh (23%)	5.2	4.1
Brewer's ale 2 + 3[b]	Fresh (27%)	3.1	1.9[a]

[a] First crop of crystals only. [b] A mixed sample.

TABLE II

LOSS OF TREHALOSE DURING AGING OF YEAST

Source	History	Trehalose in g./kg. (from rotation)
Baker's 1	Fresh	10.0
	4 Days at 20°	2.7
Baker's 3	Fresh	7.6
	6 Days at 20°	0.1
Baker's 1	Fresh	17.3
	6 Weeks at 5°	2.4
Baker's 2	Fresh	17.0
	4 Weeks at 5°	14.0
	17 Weeks at 5°	1.6
Baker's 3	Fresh	8.4
	8 Weeks at 5°	0.1
Baker's 1	Fresh	8.7
	12 Months in alcohol	8.5[a]
	15 Months in alcohol	8.4
Baker's 2	Fresh	8.9
	12 Months in alcohol	8.4[a]

[a] In each of these cases 7.7 g. of crystalline trehalose was isolated as the first crop, and identified in the usual way.

Summary

An improved method has been described for the preparation of trehalose from compressed baker's yeast. By the same method it has been found, contrary to previous investigators, that both top and bottom brewer's yeasts also may contain appreciable quantities of this sugar. Payen's recent report that "active dried yeast" is a rich source of trehalose has been confirmed.

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