4. The analysis of the boiled mixture with tryptophan present shows errors in the histidine and the cystine fractions of the bases (cystine 37.9% not precipitated), in the ammonia fractions and in the filtrate from the bases.

5. When proline is added to the mixture of 14 amino acids (no tryptophan present) and the unboiled mixture is then analyzed, errors are found both in the basic fraction and in the fractions in the filtrate from the bases. Apparently, phosphotungstic acid precipitates a part of the proline with the diamino acids. This proline nitrogen distributes itself between the arginine and the histidine fractions, and because of its entire lack of amino nitrogen, the calculations of Van Slyke's method cause the lysine fraction to show a loss.

6. When the mixture containing proline is boiled for 24 hours prior to analysis the basic nitrogen fractions still far exceed the calculated values, with a corresponding decrease in the fractions of the filtrate. Cystine again is only partially precipitated (73.3%) and the ammonia nitrogen is increased.

7. In general, the data show that both tryptophan and proline produce errors in the Van Slyke nitrogen distribution if they are present in a protein or a mixture of amino acids.

8. The cystine value of a Van Slyke analysis on a 24-hour protein hydrolysate may be taken to represent approximately 65% of the true cystine nitrogen present in the unboiled material.

ST. PAUL, MINNESOTA

[CONTRIBUTION FROM THE DEPARTMENT OF ZOÖLOGY, UNIVERSITY OF OREGON]

THE EFFECT OF CALCIUM SULFATE ON THE GROWTH AND FERMENTATION OF YEAST[1]

BY OSCAR W. RICHARDS

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While various observers have determined the effect of the chloride and the carbonate of calcium on the growth and fermentation of yeast, no one has studied the effect of the sulfate. Since the sulfate is the "permanent hardness" of water, it seemed desirable to study the effect of this salt on a pure culture[2] of *Saccharomyces cerevisiae* under controlled conditions.

The culture medium was the same as that used by Williams[3] except that the calcium chloride was omitted. The medium was made up in a concentrated solution

[1] The writer wishes to acknowledge his indebtedness to Dr. Harry Beal Torrey for helpful suggestions and criticisms throughout the investigation. He also wishes to thank Dr. H. B. Yocom and Dr. Roger Williams for critically reading the manuscript.

[2] Isolated for the writer through the courtesy of Mr. H. E. Turley of the American Bakers Association.

[3] Williams, *J. Biol. Chem.*, **42**, 260 (1920).

so that 3.5 cc. contained the nutrients needed in 10 cc. of Williams' medium. A suitable amount of a 0.01 *M* calcium sulfate solution was added to this amount of concentrated nutrients and enough water to make the final volume 10 cc. for each of the different strengths of solution used except for the 0.01 *M* solution. This was prepared by adding the solid nutrients to the stock solution of the sulfate. Such a procedure was necessary as this concentration is very close to the saturation point. These solutions were sterilized by heating for ten minutes in an autoclave at 1 atmosphere pressure. This amount of sterilization was adequate and did not affect the sugar content of the medium. The calcium sulfate and all the nutrients used except the sugar and the asparagine were Baker's analyzed C. P. materials. The cane sugar was a good commercial grade and the asparagine was Pfanstiehl's quality.

The stock yeast was grown in Williams' medium. The following calcium sulfate solutions were tested: 0.01, 0.004, 0.002, 0.0013, 0.001, 0.0004, 0.0002, 0.0001 and 0.00001 *M*. For each test calcium sulfate solutions with the nutrient materials, prepared as described above, were inoculated with 0.10 cc. of a yeast suspension taken from a three-day old culture by means of a 1cc. pipet graduated to 0.01 cc. The tubes were incubated at 30° with a variation of less than 1°. For convenience, the yeast cultures were grown in test-tubes 18 × 150 mm.

At the end of 20–24 hours the number of yeast cells in a sample volume of each of the tubes was counted by means of a Levy one-piece hemacytometer. The sample volume used was 0.04 cu. mm., or the 16 small squares of the single Neubauer ruling. Experiment showed that an even suspension of the yeast could be obtained by shaking the tube in a rotary, circular manner. For the earlier observations sixteen unit volumes were counted, and for the later observations four to eight unit volumes were counted for each tube, and the results were averaged. The probable error of the counts varied from 1 to 2%, which is sufficiently small to be ignored in this report.

The results obtained by averaging two independent series are presented in Fig. 1. Series 1 was counted at intervals for 98 hours and Series 2 was observed for 72 hours. The two series agree very well except for the counts at about 24 hours of growth. This irregularity is probably due to the fact that at this period it was very difficult to break up the colonies sufficiently to obtain an even suspension.

These results show that very small amounts of calcium sulfate are insufficient for optimum growth which occurs when the concentration is 0.0001 *M* and that greater amounts become more toxic up to the strongest concentration tested. However, after about 40 hours of growth this optimum concentration shifts to 0.0002 *M*. This shift may be due to the fact that some of the sulfate has been precipitated as the alcohol content increased, since the sulfate is less soluble in alcohol than in water. That the effect is due to the calcium rather than the sulfate ion was tested by using calcium chloride in one series. The chloride curve was very similar to the sulfate curve.

These results agree in so far as they may be compared with those of other observers who used other yeasts and other calcium salts. Fulmer, Nelson and Sherwood[4] found that calcium chloride stimulated yeast growth up to 0.1% (about 0.00013 *M*) but further additions up to 0.6% had no

[4] Fulmer, Nelson and Sherwood, THIS JOURNAL, **43**, 186 (1921).

more effect. They also found that calcium carbonate gave increasingly better growth up to 0.04% (about 0.000025 *M*) but with greater concentrations there was a decided fall in the curve. The addition of magnesium sulfate to the chloride did not improve their results. Mitra[5] found that the chloride stimulated growth for the yeast *Saccharomyces ellipsoideus* up to a concentration of 0.1 *M* and that further additions of the salt were toxic.

The hydrogen-ion concentration changed uniformly during the growth period to P_H 3.0 which makes it a constant factor in the tests. The Sörensen value (P_H) was determined by the drop method[6] using commercial standards.[7]

In order to compare this optimum calcium sulfate concentration with the concentration of the salt found in city water supplies the analyses of the water supply from 15 cities from different parts of the United States were collected and averaged. The average calcium concentration of these different sources of water supply was 0.001466 *M* with a range of from 0.0021 to 0.0002 *M*. One analysis showed that the calcium content for one day in one city was 0.0054 due to an abnormal working of the purification system. While this was not included in the above average, it is significant from the baker's standpoint. The calcium sulfate concentration seems to be not greater than about 0.0001818 *M* for about one-third of the cities which distinguished between calcium and the different calcium salts.

The fermentation tests were made with Einhorn saccharometers using 1% sugar solution inoculated with 1.00 cc. of a three-day-old suspension of the stock yeasts. Each test consisted of from two to five control tubes and from five to ten tubes of one calcium sulfate solution prepared in the same way as the growth tubes. These tubes were incubated at 30°. The small errors due to the fact that different places on the incubator shelf had slightly different temperatures were eliminated by placing the control and mineral tubes in the same places in every test. Readings of the number of cc. of carbon dioxide produced were made at frequent intervals until the maximum was obtained. The average fermentation period was about 48 hours. Then the production of gas in the control tubes was averaged and from this average the average production of gas in the calcium sulfate tubes of that test was subtracted. Since the gas production of these tubes was always less than that of the control tubes, this could be done for the entire range of salt concentrations tested. This method gave values for all the series that were comparable with one another and independent of slight variations in the density of the seed-yeast suspension, etc.

[5] Mitra, *Univ. California Pub. Agr. Sci.*, **3**, 63–102 (1917).

[6] Felton, *J. Biol. Chem.*, **46**, 299 (1921).

[7] Buffer tablets from Pyrolectric Instrument Co., Trenton, N. J.

The results of the fermentation tests, shown by the broken line in Fig. 1, were much the same as those of the growth tests. The optimum con-

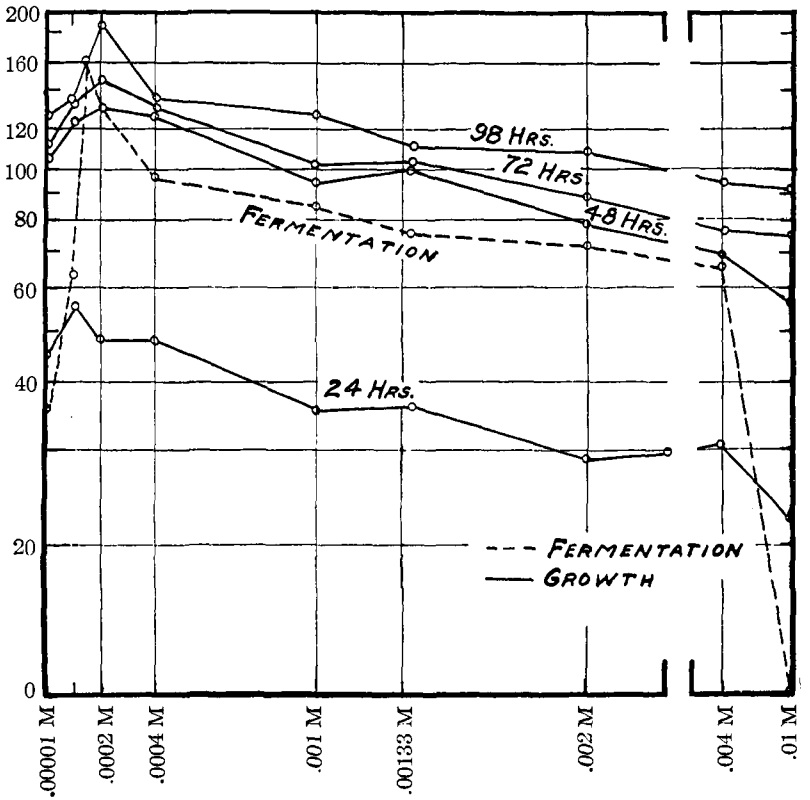

Fig. 1.

centration seems to be at 0.00013 M or at a slightly less concentration than the growth tests. In order to make sure of this a few growth tests were

TABLE I

GROWTH OF THE YEAST IN CALCIUM SULFATE SOLUTIONS

Concn. M	Average number of yeast cells in 0.04 cu. mm. Series 1 hours 20	27	48	72	98	Series 2 hours 24	50	72
0.01	14	37	71	84	90	19	22	64
.004	24	46	78	83	94	21	65	70
.002	20	44	90	95	109	23	71	82
.0013	25	48	100	104	112	..	...	...
.001	26	53	106	110	129	26	84	101
.0004	28	68	128	131	137	..	...	...
.0002	26	84	141	160	188	38	124	130
.0001	30	104	130	135	136	37	118	120
.00001	28	66	99	110	125	39	110	117

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tube bubbled through the lime water so fast that it was not so completely absorbed by the lime water. Thus, the effect of the sulfate is the same during a short fermentation period as it is during the maximum fermentation period.

These results lead to the conclusion that the optimum calcium sulfate concentration for fermentation is the same as, or perhaps very slightly less than, the optimum for growth. Most water supplies furnish more calcium than, this optimum. This may not be unfortunate, however, because much larger concentrations of the salt seem to be desirable in baking,[8] and improvers containing considerable amounts of the sulfate, such as the Arcady,[9] are frequently added to the doughs. This increased tolerance is probably due to some buffer action.

Summary

It was found that the best concentration of calcium sulfate for the most efficient growth and fermentation of the yeast *S. cerevisiae* is at about 0.0001 *M*. Higher concentrations of the salt inhibit growth and fermentation, and lower concentrations are inadequate for best growth and fermentation. Growth was studied by examination of a synthetic culture medium over various periods of time up to 98 hours. Fermentation was studied during both short and maximum periods of time. A study of the calcium sulfate content of water supplies shows that the concentration is usually greater than the optimum indicated above for the yeast and may occasionally be fifty times as great.

EUGENE, OREGON

[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT, UNIVERSITY OF BRISTOL]

ATTEMPTS TO SYNTHESIZE MYRICETIN

BY H. F. DEAN AND M. NIERENSTEIN

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In the present communication we describe a series of experiments to synthesize myricetin (I) and although we have failed in these attempts our results establish the interesting fact that the method of Kostanecki for the synthesis of the flavanols so far generally applicable breaks down in the case of myricetin.

Following Kostanecki's procedure we have prepared 2-hydroxy-4,6,-3′,4′,5′-pentamethoxy-chalcone (II), from which we have obtained 4,6,-3′,4′,5′-pentamethoxy-flavanone (III). However, all our attempts to prepare the corresponding *iso*nitroso-flavanone, which would have ultimately yielded myricetin (I), have only led to negative results.

[8] Kohman and others, *J. Ind. Eng. Chem.*, **8**, 781 (1916).

[9] *Connecticut Agr. Exp. Sta. Rept., Bull.*, **200** (1917).