produced at lower temperatures provided other factors are favorable. A contributing factor is that higher coefficients would have been obtained had a temperature value been available for meal 5676.

The relatively high positive correlation of most of the methods with solubility of nitrogen in dilute sodium chloride indicates that this constant is of value in measuring protein quality. However, Lyman (*7*) found certain meals had extremely low protein solubility, and yet had excellent protein quality as determined by both chick and rat feeding tests.

Evaluation of Rat Repletion Method

The principal criticism of the rat repletion method, based on these experiments, is failure to give the desired precision. This, of course, is a general fault with protein quality measurement. Another objection is the necessary use of much arbitrary technique because optimum points in the method have not been fully established—for example, degree of depletion of the rats, length of depletion period, and daily level of nitrogen. Frost and Sandy (*4*) found that more significant differences could be obtained with a level of 0.24 gram of nitrogen than with 0.12 gram, but this did not establish an optimum level. It is possible that precision could be improved with a higher level of nitrogen than used here with these meals.

Because the test products and the reference protein are fed at the same nitrogen level, determination of nitrogen in both the test products and the reference protein is necessary. The method has the following marked advantages.

1. Effects of palatability of diet are eliminated. 2. Effects of unequal protein intake are avoided. 3. Advantages of economy of time—rats may be prepared for assay usually in three weeks, the actual assay requiring only 10 days. Because it is not necessary to use rats of exact weight and age, the work can be made to fit into other schedules. 4. A relatively small amount of sample is required, equivalent to approximately 12.5 grams of nitrogen. 5. The same rats may be used several times with no more than a 10-day interval between tests.

Summary

Data are reported from the application of a rat repletion method to estimation of protein quality in 12 cottonseed meals used in a collaborative study of the effects of processing methods on the nutritive properties of cottonseed meal. Mean gain in body weight by groups of protein depleted rats during a 10-day repletion period was used as the criterion of response for estimating protein quality. The meals were tested in four feeding trials and mean gains on the 12 meal samples ranged from 13.6 to 28.8 grams with calculated least significant mean differences (P = 0.05) ranging from 2.7 to 3.6 grams. Results with the rat repletion method are compared with those obtained on the same samples by other laboratories using other biological methods.

Data are presented to show correlation existing between the rat repletion method and other measurements of protein quality. Significant negative correlations were found between the rat repletion method and bound gossypol content of the meals. Negative correlation between all protein quality methods and free gossypol, though not statistically significant for any one method, indicates that free gossypol in amounts below 0.05% has an adverse effect on growth.

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FERMENTATION ACCELERATOR

Dried Activated Sludge as a Fermentation Accelerator

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IT HAS BEEN FOUND that dried activated sludge and several preparations derived from this material are activators in the fermentation of various sugars to ethyl alcohol by yeast. (The material studied in detail here was the dried activated sludge produced by the Sewerage Commission of the City of Milwaukee at its plant in Milwaukee, Wis., and sold under the trade name of Milorganite.) The addition of comparatively small amounts of these activators results in a considerable decrease in the fermentation time and, in many cases, in an increase in the amount of carbon dioxide evolved. It is this acceleration of the rate of fermentation which is designated as "activation."

Dried activated sludge has not heretofore been used as a constituent of fermentation media either as a source of nutrients or activators for fermentations by yeast. There have been a number of preparations described previously for promoting or activating a yeast fermentation. Typical materials discussed are an aqueous extract of soybeans (*14*), acid or alkaline hydrolyzates of scleroproteins (*6*), corn and wheat proteins (*7*), alkaline earth hydroxides (*11*), phosphoric acid hydrolyzates of protein (*8*), acid hydrolyzates of animal protein (*2*), bran infusion (*4*), activated char containing protein material in its pores (*9*), activated carbon (*15*), amino acids and proteins (*13, 27*), ethanolamine (*26*), sodium salts of the fatty acids (*10*), an aqueous corn extract (*25*), an aqueous extract of liver (*24*), and a cozymase derivative (*12*). These activating substances have been known to the fermentation industry for some time, yet none of these has found application in industrial practice. This is probably due to the fact that many are inactive in small amounts, and the large amounts necessary to give the desired effect introduce a prohibitive cost. Also many of these substances are activators when the yeast is fermenting pure sugar solutions, and they no longer show activation if introduced into grain or molasses mashes used industrially. In

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Dried activated sludge has been shown to increase the rate of fermentation of molasses, grain, or synthetic substrates by yeast. The acceleration is produced by small amounts (0.01 to 0.1% based on the weight of the mash) and is especially marked in the early stages of the fermentation. The time necessary to complete a fermentation is markedly shortened and, in the laboratory, increased yields of carbon dioxide and alcohol are obtained. Preliminary experiments aimed at concentrating the accelerating factor are described. The results indicated that the "fermentation factor" and vitamin B_{12} are not identical.

some cases use of the activator results in an increase in yeast cells at the expense of the desired ethanol.

The presence of vitamin B_1 has been shown to be necessary to produce rapid fermentation rates (*18, 19*). In pure sugar solution additions of vitamin B_1 will produce increased rates of fermentation. Since grain and molasses mashes as prepared in the plant or laboratory usually contain sufficient amounts of vitamin B_1, addition of the vitamin to such mashes does not cause an acceleration of fermentation.

In the work described, Milorganite produced significant activation in the fermentation of sugars by yeast. The activation takes place whether dried activated sludge (or a preparation derived from this material) is added to malt wort, pure sugar solutions containing adequate amounts of minerals and vitamins, molasses mashes, or converted grain mashes. These increased fermentation rates do not involve increases in yeast cells at the expense of alcohol.

The activated sludge process is a descriptive term given to a particular method of sewage purification. In this process (*3*), sewage, industrial and/or domestic, can be converted to a form whereby the original solids content and B.O.D. of the aqueous phase are reduced to small fractions of the original. The solids in the aqueous phase can be recovered and processed to give a material of important fertilizer value.

Experimental Methods

The assumption that the intense aerobic fermentation by the comparatively large group of organisms involved in the activated sludge process could produce factors or nutrients of value for the now known industrial fermentations seemed quite reasonable. For several reasons we turned to the alcoholic fermentation of carbohydrates by yeast as the screening assay. With yeasts we were not restricted to complex media or completely sterile equipment. In addition, the course of the rate of fermentation could be followed easily with comparatively simple equipment. Most of the work was done by following the course of a fermentation gravimetrically, losses in carbon dioxide being determined.

The production of carbon dioxide was measured by determining the loss in weight of the fermenting mash. In preliminary experiments the determinations were made in 125-ml. Erlenmeyer flasks fitted with calcium chloride tubes fitted with Bunsen valves to allow for the escape of the carbon dioxide. Actually only the bulb of the calcium chloride tube was used; this was filled with anhydrous calcium chloride to trap both the water vapor and the ethanol as they are carried out with the escaping carbon dioxide. The flasks were charged with 50.0 ml. of the substrate and inoculated with a given amount of yeast. For most of these experiments ordinary baker's yeast was used, either in the cake or as the dried active form. The yeast used was uniformly suspended in the distilled water and added by pipette to the test mash. The flasks were shaken continuously while they were immersed in the constant temperature bath. For weight determinations an analytical balance was used with weights taken to the nearest milligram. Readings were taken at intervals with the time of inoculation being zero time. This method has been used by other investigators in the fermentation field (*21, 23*).

The procedure described above was designated as the small-scale procedure. To duplicate more nearly a commercial alcohol fermentation the following "large-scale" procedure was used. About 220 grams of molasses was dissolved in 900 ml. of tap water; 0.5 to 1.0 gram each of ammonium sulfate and ammonium acid phosphate added; the pH of the solution adjusted to 4.9 with sulfuric acid; and the solution diluted to exactly 1000 ml. A 960-ml. aliquot of the mash was transferred to a 2-liter fermentation flask; the remaining 40 ml. was placed in an Erlenmeyer flask. Both were plugged with cotton and were sterilized at 15 lb. pressure for 15 minutes. (Some experiments were run with no sterilization.) On cooling the additive being tested was added to the Erlenmeyer flask; 5.00 ml. of a 50.0-ml. aqueous suspension of 0.50 gram of baker's yeast was added immediately thereafter. The small flask was fitted with a calcium chloride tube carrying a Bunsen valve, weighed, and then incubated at 31° C. for 16 to 20 hours. After this period the flask was again weighed and the contents transferred to the large fermentation flask which was then fitted with a calcium chloride tube and Bunsen valve. The rate of fermentation was followed by determining the loss in weight due to the carbon dioxide evolved. The flasks were incubated at 31.5 ± 0.5° C. during the fermentation. These flasks were shaken by hand at intervals during the course of fermentation to avoid effects due to inert particles.

The degree of activation for these fermentations was determined in several ways. For qualitative comparisons fermentations were run and readings were

Table I. Effect of Dried Activated Sludge on Rate of Molasses Fermentation

Sample	Dried Activated Sludge Added (Wt./Vol.), %[a]	Loss in Weight (Grams) Due to CO_2 Evolved After				
		2 Hr.	3.5 Hr.	5 Hr.	6.5 Hr.	8 Hr.
0	0 (controls)	0.041	0.161	0.368	0.653	1.040
1	0.002	0.073	0.213	0.469	0.816	1.326
2	0.010	0.083	0.260	0.550	0.995	1.510
3	0.020	0.092	0.283	0.585	1.092	1.610

[a] Ground dried activated sludge with particles passing 150 mesh.

Table II. Effects of Particle Size of Dried Activated Sludge on Molasses Fermentation Rate

Sample	Particle Size (Screen Mesh)	Theoretical CO_2[a] Evolved (%) After								Hours Required for 83% of Theoretical CO_2 to Be Evolved
		5 Hr.	9 Hr.	23 Hr.	27 Hr.	31 Hr.	49 Hr.	55 Hr.	72 Hr.	
1	Control (none)	1.6	11.0	59.5	65.7	71.2	81.4	81.8	83.5	46
2[b]	On 80	7.3	31.9	75.5	80.6	82.2	83.5	83.6	84.2	33
3	80–200	8.1	32.1	76.0	81.4	83.3	86.5	87.0	87.0	33
4	200–270	16.0	39.4	83.3	88.4	90.0	91.6	91.9	93.2	23
5	270–325	16.0	37.8	81.0	86.7	90.7	93.5	94.1	95.0	23
6	Pass 325	16.0	37.8	83.0	89.2	91.0	92.7	93.4	93.6	23

[a] Theoretical = 61.7 grams.
[b] 2.0% (wt./vol.) of dried activated sludge added to each fermentation flask.

taken at regular intervals. Those fermentation flasks to which a material possessing the activator had been added usually showed that an amount of carbon dioxide greater than that shown by the control had been liberated. Tabulating or graphing these data gave a comparative picture of the effect of the addition of the activator. For quantitative comparison two methods were used. Taking some particular fermentation period, usually 4 or 6 hours, the difference in the amounts of carbon dioxide evolved between the control flask and the activated flask was determined and denoted as ΔCO_2. Since the control fermentation rate, and therefore the amount of carbon dioxide evolved by the control, varied from experiment to experiment, the ΔCO_2 also varied; therefore, the percentage activation was often used. Percentage activation was calculated as follows: $\Delta CO_2/CO_2$ of control $\times$ 100. This type of calculation was used only for the small scale experiment. For the large scale experiment a quantitative comparison of the effect of the activators was determined by graphing percentage of theoretical carbon dioxide evolved versus time and determining the time required to evolve an arbitrary amount of carbon dioxide, usually 83% of the theoretical carbon dioxide.

Dried Activated Sludge as Activator In Molasses Fermentation

While most of our introductory work was done with extracts of dried activated sludge, it was not long before it was determined that Milorganite itself appeared to be a potent activator. Typical results are illustrated by the following experiment.

The substrate was prepared so that each fermentation flask contained 12.5 grams Cuban blackstrap molasses, 0.05 gram of ammonium sulfate and 0.05 gram of potassium dihydrogen phosphate in 50.0 ml. of tap water with the pH at 5.0. Each flask was inoculated with 150 mg. of dried active yeast in aqueous suspension (2.0 ml.), see Table I. (Values quoted in the tables are the averages of duplicate or triplicate runs.)

Particle size was suspected of having some effect in the activation, and this was borne out in an experiment where Milorganite fines were fractionated on a series of sieves and each fraction was added at the level of 20 grams per liter of mash (2.0% weight to volume); 0.5 grams of the activator were added to the seed mash, the remainder of the activator being added to the 960 ml. of mash at the time the seed mash was transferred to it (see Table II).

All the various fractions gave increased fermentation rates. These sieved fractions fell roughly into two groups: one group of mesh size greater than that so as to be retained on a 200-mesh screen; the other group being of a size so as to pass a 200-mesh screen. The first group reduced the fermentation time to about two thirds that of the control while the latter group reduced the time to half that of the control value. Of particular interest is the considerable increase in yield shown by samples 4, 5, and 6.

After it was determined that the particles able to pass a 200-mesh screen were the best activators, this group (275–325 mesh) was studied further to determine the effect of decreased concentrations of fine dried activated sludge on the fermentation rate. These experiments revealed that additions of ground dried activated sludge as low as 0.50 grams per liter of mash (0.05% weight to volume) produced significant increases in fermentation rates. It was also indicated that dried activated sludge particles which passed a 140-mesh screen were almost as active as those particles of smaller mesh. To determine how little dried activated sludge could be added to an alcohol fermentation and still produce an increase in rate an experiment was run in which the amount of dried activated sludge added was reduced to 1 mg. per liter of mash (Table III).

The addition of as little as 0.0001% of dried activated sludge showed an activating effect on the fermentation rate. Increasing amounts gave increased rates; additions of amounts greater than 0.0050% appeared to have no greater effect. The yield increase was again noted here.

Effect of Dried Activated Sludge When Molasses Concentration Is Greatly Increased. It is well known in the fermentation industry that an increase in the sugar concentration in the mash to be fermented by yeast causes a decrease in the fermentation rate, often

Table III. Effect of Small Amounts of Dried Activated Sludge on Molasses Fermentation Rates

Sample	Dried Activated Sludge Added (Wt./Vol.), %[a]	Theoretical CO_2 Evolved (%) After							Hours Required for 83% of Theoretical CO_2 to Be Evolved[b]
		8 Hr.	24 Hr.	28 Hr.	32 Hr.	48 Hr.	56 Hr.	72 Hr.	
1	0.00 (Control)	4.5	65.0	73.2	77.4	82.8	83.6	83.6	50
2	0.0001	11.7	70.0	76.0	81.2	83.8	84.4	85.2	46
3	0.001	13.6	70.7	76.8	81.7	84.5	84.8	85.5	40
4	0.005	14.2	71.5	77.8	83.4	86.5	86.5	87.1	30
5	0.010	18.4	77.5	82.5	83.4	86.5	86.5	86.5	30
6	0.020	16.2	74.6	80.0	84.6	86.8	87.3	87.8	29
7	0.030	16.7	74.0	81.3	84.7	85.4	85.4	86.0	29
8	0.040	17.7	74.0	80.5	84.0	86.1	86.1	86.5	29
9	0.050	18.3	75.2	80.7	84.6	85.8	85.9	86.1	29

[a] >140 mesh.
[b] From a graph of these data, the time interval necessary for completion of the fermentation was obtained.

Table IV. Effect of Fine Dried Activated Sludge in Alcohol Fermentation of Molasses Mashes of Increased Concentration

Sample	Dried Activated Sludge Added (Wt./Vol.), %	Molasses/ Liter of Mash, Grams	Theoretical CO_2 Evolved (%) After 19 Hr.	24 Hr.	27 Hr.	43 Hr.	48 Hr.	72 Hr.	Hours Required for 83% of Theoretical CO_2 to Be Evolved
0	0 (Control)	213[a]	47.0	59.3	65.5	80.7	82.5	84.5	49
1	1.5	213	57.0	69.0	75.2	85.5	86.5	86.7	29
2	0 (Control)	298[b]	36.4	45.9	51.1	69.3	73.1	85.7	63
3	1.5	298	47.0	56.2	60.2	79.3	83.1	89.2	48
4	0 (Control)	341[c]	31.7	33.5	47.4	61.8	65.7	79.0	>75
5	1.5	341	44.6	51.7	54.8	68.4	72.0	85.7	>72

[a] Theoretical CO_2 evolved = 61.7 g.
[b] Theoretical CO_2 evolved = 85.2 g.
[c] Theoretical CO_2 evolved = 98.3 g.

along with a decrease in the yield of alcohol obtainable. These effects are due to the unfavorable osmotic pressure exerted by mashes of high sugar concentration and by the relatively large concentrations of alcohol built up in solution after the sugars have been converted to alcohol. In several experiments it has been shown that the addition of fine-ground dried activated sludge to a mash containing up to 40% more molasses than that usually recommended results in a fermentation rate comparable to the rate shown in a mash of the lower and usual molasses concentration. Even when the molasses concentration was increased 60% and the rate of fermentation of the control had slowed down to an "impractical" rate, additions of dried activated sludge increased this rate considerably. Typical results which have been obtained using the large scale procedure outlined are given in Table IV.

With samples 0, 2, and 4, to which no dried activated sludge was added, increased concentrations of molasses gave decreased fermentation rates. In every case in which dried activated sludge was added an increased rate over that of the comparative control was obtained. These results indicated that the molasses concentration can be increased by as much as 40% in the presence of the activator and still allow a fermentation rate equal to or greater than that shown by the usual 213 gram mash containing no activator. It is interesting to note that the final alcohol concentration for the 213-gram sample was determined as being 6.60% by volume, for the 298-gram sample the alcohol concentration was 9.35% by volume, and for the 341-gram sample the alcohol concentration reached 11.3%.

For these experiments a comparatively high amount of dried activated sludge was used. The effect of smaller amounts of activator in molasses mashes of higher concentration has not as yet been determined.

A similar series of experiments was run using citrus molasses instead of cane molasses as the substrate. The results obtained were practically identical with those obtained previously; a large increase in fermentation rate as well as an increase in alcohol yield was noted.

Activity of Dried Activated Sludge And Its Aqueous Extract in Grain Alcohol Fermentation

Preliminary experiments in this laboratory had indicated that additions of dried activated sludge to a mash prepared from grain (corn) would accelerate the fermentation rate. In contrast to molasses mashes, it is difficult if not impossible to duplicate a plant-prepared mash entirely in a laboratory away from a plant. To evaluate more exactly the effect of dried activated sludge in grain mashes, experiments were run at the laboratories and plant of The Grain Processing Corp. in Muscatine, Iowa.

The procedure used in these experiments was essentially that described for the large-scale laboratory tests described above; however, a number of details were varied so the exact procedure is given below:

A number of 500-ml. Erlenmeyer flasks were fitted with a rubber stopper carrying a calcium chloride tube fitted with a Bunsen valve. To these flasks there were added 100 ml. of a corn mash which had just been inoculated with the strain of yeast used in the plant. This inoculation had been done in the regular plant equipment. To several of the flasks varying amounts of the activator were added, others contained only mash, and some were used as controls. The flasks were then weighed and placed in a constant temperature room at 30° ± 0.5° C. After 21 hours in this room, the flasks were weighed, and 20 ml. of this fermented mash was used to inoculate the large fermenters.

The substrate for these fermenters was prepared as follows: 22 liters of gelatinized corn was withdrawn from a plant line and placed in a large milk can. The temperature of the mixture was lowered from 180° F. to 135° F. in a stream of cold water. At this temperature 2 liters of a solution of fungal amylase was added and the well-stirred mixture allowed to stand one half hour. One thousand milliliter portions of the converted mash were placed in the 2-liter fermentation bottles and inoculated with 20 ml. of the corresponding mash from the 500-ml. Erlenmeyers. To fermenters being inoculated with mash containing no dried activated sludge varying amounts of dried activated sludge were added. Wherever the additive was present in the seed mash, no further amounts were added to the large fermenter. These flasks were also covered with a stopper containing the calcium chloride tube and Bunsen valve, weighed, and incubated in the constant temperature room. The course of the fermentation was followed as usual by weighing the flasks at intervals and noting the loss in weight due to the carbon dioxide evolved.

Table V. Effect of Dried Activated Sludge Added to Fermentor in Grain Fermentation

Sample	Dried Activated Sludge Added (Wt./Vol.), %	Loss in Weight (Grams) Due to CO_2 Evolved After 18.3 Hr.	23.1 Hr.	26.6 Hr.	43.0 Hr.	50.5 Hr.	67.5 Hr.	Hours Required for 75.3 G. Theoretical CO_2 to Be Evolved
0	0 (Control)	40.7	50.8	55.5	68.6	70.1	75.3	67.5
9	0.025	45.9	53.4	56.7	68.9	72.3	76.3	60.0
10	0.050	47.0	53.9	57.8	69.6	72.1	77.1	60.0
11	0.10	48.8	55.5	60.2	69.9	72.2	76.6	57.5
12	0.25	49.2	55.8	58.0	70.0	73.4	76.5	57.5
13	0.50	50.6	56.3	59.0	71.0	74.4	77.2	55.0
14	1.00	50.7	56.1	59.7	70.3	73.0	77.4	55.0

In this series of experiments the relative effect of adding varying amounts of dried activated sludge to the corn mash was determined.

In the experiment given below, dried activated sludge was added only to the fermenters. The seed mash flasks were identical with the controls and they evolved essentially the same amount of carbon dioxide (Table V).

These data were plotted in the usual manner. If we consider 67.5 hours as the end of the fermentation and 75.3 grams of carbon dioxide as the point of reference, the time necessary to evolve this 75.3 grams of carbon dioxide in the activated fermentation was determined.

With 1000 mg. per liter (0.1% weight to volume) there is a 10-hour reduction in time corresponding to a 14.8% reduction. Similar results were obtained when the dried activated sludge was added directly to the seed mash.

Effect of Dried Activated Sludge on Fermentation Rate of a "Complete" Synthetic Substrate

Having determined that Milorganite was capable of accelerating the rate of fermentation of several types of molasses mashes it became imperative to determine whether the dried activated sludge was supplying some known nutrient in which the mashes used were deficient or whether the activator was supplying some new factor.

A large number of substances have been shown to be essential for maximum yeast growth and maximum fermentation rates. From the materials quoted as being essential by the various investigators (*17*, *20*), a composite substrate containing all the organic and inorganic substances mentioned in these references was formulated. It was determined that some of these reagents had no effect on fermentation rates and these were omitted in further experiments. The substrate which was finally used in these experiments was prepared as follows:

Solution A	Grams
KH_2PO_4	40.0
KCl	17.0
$MgSO_4.7H_2O$	5.0
$CaCl_2.2H_2O$	5.0
$MnSO_4.4H_2O$	0.10
$FeSO_4.7H_2O$	0.10
$(NH_4)_2SO_4$	100.0
$(NH_4)_2HPO_4$	40.0
H_3BO_3	0.020
$ZnSO_4$	0.020
$CuSO_4.5H_2O$	0.002
KI	0.002

The ingredients were dissolved in distilled water; the pH adjusted between 5.0 and 5.2 with sulfuric acid, and the solution diluted to 2000 ml. with distilled water.

Solution B	Milligrams
Thiamin	100
Pyridoxine	10
Inositol	100
Nicotinic acid	100
Riboflavin	100
Aspargine	100
Aspartic acid	200
Folic acid	100
Biotin	0.5

These materials were dissolved in 900 ml. of distilled water; the pH was adjusted to 5.0 and the solution diluted to 1000 ml.

Solution C was an acid hydrolyzate of casein containing 100 grams of solids per 100 ml. of solution (obtained from General Biochemicals, Inc., Chagrin Falls, Ohio).

The substrate was prepared then by adding 50.0 grams of C.P. sucrose, 100 ml. of solution A, 50 ml. of solution B, and 40 ml. of solution C, shaking to produce a homogeneous mixture, and diluting to 1000 ml. with distilled water; 50.0-ml. aliquots were placed in the small fermentation flasks. Further pH adjustment usually was not required. Dextrose could replace the sucrose without altering the type of result obtained.

The first experiments using this "synthetic" substrate indicated that additions of dried activated sludge to it would result in an increase in the fermentation rate. Using the small-scale procedure on 50.0 ml. of substrate inoculated with 200 mg. of baker's yeast the results shown in Table VI were obtained.

With the increasing amounts of dried activated sludge the fermentation rate was increased. The largest amount of activator added was 500 mg., but even with this amount it appeared that the maximum possible degree of activation had not been achieved. Another experiment was run in which larger amounts of dried activated sludge were added to the standard substrate.

The addition of as much as 4.0% (2.0 grams) of dried activated sludge gave an increasing amount of activation but even at this level maximum activitation had not been attained.

From these results it was indicated that the dried activated sludge was supplying some unknown factor or factors to the yeast.

Table VI. Effect of Small Amounts of Dried Activated Sludge on Fermentation Rate of Synthetic Substrate

Sample	Dried Activated Sludge Added (Wt./Vol.), %	Loss in Weight (Grams) Due to CO_2 Evolved After 2 Hr.	4 Hr.	5 Hr.	6 Hr.	7 Hr.	8 Hr.
0	0 (control)	0.049	0.174	0.301	0.444	0.572	0.701
1	0.02	0.066	0.246	0.356	0.494	0.647	0.804
2	0.10	0.068	0.260	0.376	0.523	0.670	0.820
3	0.40	0.067	0.284	0.417	0.590	0.747	0.903
4	1.00	0.071	0.313	0.473	0.674	0.841	0.966

Some Indicated Properties of the Factor

Many results had indicated that an acid hydrolyzate derived from dried activated sludge would show a high degree of activity, often similar to that of dried activated sludge itself. The alkaline hydrolyzates on the other hand showed only a fraction of the activity shown by the dried activated sludge indicating some destruction of the factor. While the acid hydrolyzate is active, we have no indication as to whether the activity is due to a material identical with that found in dried activated sludge or whether the activity is due to some altered form of the original fermentation factor.

Preliminary experiments had shown that the factor is extractable by water; the extraction, however, is incomplete since the residue shows an appreciable degree of activity, and the extract itself is no more potent on a weight-for-weight basis than the original dried activated sludge. In these preliminary experiments there was also determined the effect of temperature on the activity of the aqueous extract obtained. It was felt, however, that aqueous extraction was the mildest type of treatment and should give solutions containing the factor in unaltered form or in the same

Table VII. Activity of Aqueous Extracts of Dried Activated Sludge on Molasses Mash

Sample	Extraction Temperature	Solids Added	Weight Loss (Grams) Due to CO_2 Evolved After 2 Hr.	3.5 Hr.	5 Hr.	7 Hr.	10 Hr.
0	..	0 (Control)	0.015	0.063	0.161	0.420	1.07
1	..	100 mg. Milorganite	0.017	0.085	0.205	0.520	1.28
2	30° C.	109 mg. extract	0.017	0.072	0.195	0.488	1.11
3	45° C.	109 mg. extract	0.018	0.077	0.206	0.520	1.22
4	80° C.	113 mg. extract	0.018	0.082	0.205	0.513	1.22

Table VIII. Activity of an Aqueous Extract of Dried Activated Sludge in the Fermentation of Molasses

Sample	Extract Added, γ	Activation, % A	B	C	Av.
1	1.0	3.8	4.6	3.1	3.8
2	5.0	9.3	8.8	5.4	7.8
3	10.0	8.2	7.9	10.5	9.0
4	50.00	11.0	7.9	11.1	10.0
5	100	11.0	11.8	12.0	11.6
6	1000	11.9	12.9	...	12.4
7	10000	15.2	15.0	...	15.1
8	100,000	21.4	21.0	...	21.2
	Milorganite Added, γ				
9	1000	13.4	15.2	...	...
10	10000	...	15.2	...	...

form in which it is available to the yeast cell.

To prepare the aqueous extracts the following procedure was used. In a 2-liter flask fitted with stirrer and condenser was placed 1 liter of water warmed to 30° C. One hundred grams of ground Milorganite was added and the mixture kept at 30° to 32° C. for 13 hours with stirring. The pH of the reaction mixture was adjusted to 7.0 ± 0.5 and kept there by additions of equal amounts of ammonia and water. The mixture was centrifuged to give a supernatant liquid which still contained some suspended colloidal matter. This colloid was removed by filtration using a small amount of Filter-Cel. The filtrate was used directly in the test procedure. Extractions were repeated at 45° C. and at 80° to 90° C. The yield of soluble solids for each of these treatments was 9.4, 11.2, and 17.8%, the highest temperature giving the highest yield.

The relative activities of each of the preparations was determined using the small-scale procedure in molasses substrate fortified with ammonium sulfate and ammonium acid phosphate. A sample of the Milorganite from which these extracts were prepared was included (see Table VII).

This and similar experiments indicated that the higher temperature extractions gave somewhat more active extracts. Since the higher temperatures also gave higher yields, further work was carried out using preparations prepared at 80° to 90° C.

Using the molasses mash it was determined that microgram quantities of these extracts would produce a consistent and significant degree of activation. Thus, an extract prepared at 90° C. and tested in three experiments over the range 1 to 100,000 γ gave the results shown in Table VIII.

If the logarithms of the weights of the extract added were plotted against the percentage activation, they would fall on a straight line. The slope of the line obtained from a graph of the average activations was not as steep as one would desire. However, it offered hope of a possible means of comparing the activities of various preparations.

Experiments indicated that the activator was stable to heat—for example, preparations which had been dried for 2 to 5 days at 70° to 80° C. appeared to be as potent as the solids in the original solution. This material (M171) was assayed several times using the small-scale procedure and using the synthetic substrate outlined above. It showed a somewhat greater degree of activity than did the preparation discussed above.

An ethanol extract was prepared by boiling 10 grams of the dry, powdered, aqueous extract (M171) under reflux for 2 hours with 100 ml. of No. 30 ethanol, filtering, washing the residue with alcohol, and repeating the extraction with the residue under reflux. This was done three times; the last filtrate was light in color, in contrast to the first which was a dark brown. The combined filtrates and washings were evaporated to dryness to yield 2.1 grams of a brown solid.

A butanol extract was prepared by stirring 2.5 grams of the powdered aqueous extract for 8 hours with 100 ml. of butanol. The butanol was replaced by 100 ml. of fresh alcohol and stirred with the powder overnight. The mixture was filtered, the residue washed with 50 ml. of butanol, the filtrate and washings combined and evaporated in vacuo to dryness. This butanol-soluble fraction was taken up in water and again evaporated in vacuo to remove the last traces of alcohol. The butanol-insoluble residue was taken up in water and evaporated to dryness in vacuo. By this procedure there were obtained 0.41 grams of the butanol-soluble fraction and 2.00 grams of the insoluble fraction.

Treatment of the dried aqueous extract with 2-propanol seemingly gives an active alcohol-soluble fraction. Two and one half grams of the powdered water extract was extracted continuously in a modified Soxhlet-type extractor for 12 hours. Color was removed only slowly from the solid; the alcohol solution after 12 hours was light orange in color in contrast to the red solution obtained with ethanol and light yellow with butanol. After removal of the alcohol by distillation in vacuo, and water, and redrying in vacuo there were obtained 2.11 grams of the insoluble material and 0.30 grams of solubles.

These various samples were assayed by the small-scale procedure using the synthetic medium and correlating activity with percentage activation at 6.5 hours. Dried active yeast was used for inoculation.

The results in Table IX are the average of two or three separate runs in which each level was run in duplicate. Each result then is the average of 4 or 6 flasks.

If the best lines through these points were determined by the method of least squares, lines of somewhat varying slope were obtained. However, to obtain an approximation of the varying degree of activity in these samples, least-square lines were obtained with the slope fixed to that of the standard aqueous extract. Using these lines and taking the point of 20% activation for reference the amount of extract necessary to produce this

Table IX. Activities of a Standard Aqueous Extract of Dried Activated Sludge and Preparations Derived from This Extract on Synthetic Substrate

Sample	Extract Added/ 50 Ml., γ	% Activation at 6.5 Hr. Standard aqueous extract	Soluble in Ethanol-	2-Propanol-	Butanol-	Insoluble in Ethanol-	2-Propanol-	Butanol-
1	0.2	7.4	11.1	10.1	4.3	3.5	0	10.9
2	1.0	10.0	14.1	8.0	7.2	4.6	0	12.1
3	5.0	13.9	17.9	19.1	3.6	2.3	0	14.4
4	25.0	19.1	21.1	31.4	...	...	0	17.2
5	100	20.6	28.1	28.8	3.6	0.6	0.4	19.6
6	1000	25.7	31.3	37.0	...	2.2	1.0	36.1
7	5000	...	...	38.0	...	5.2	17.2	...
8	10,000	30.9	35.0	44.0	3.1	...	15.5	37.6
9	50,000	36.4	42.0	...	9.5	9.4	...	...

activation could be read from the graph. These were: aqueous extract, 75 γ; butanol-insoluble fraction, 17.5 γ; ethanol-soluble fraction, 10 γ; and the 2-propanol-soluble fraction, 1.5 γ. Thus, the greatest degree of concentration of the concentrate had been obtained with 2-propanol alcohol.

We have some evidence that the factor can be absorbed on activated carbon (Norit SV) and eluted with dilute ammonia; however, we have not fully investigated this means of purification.

One experiment was run using an enzyme decomposition product of dried activated sludge. It has been known for some time that dried activated sludge is very resistant to enzymic hydrolysis. This was verified in these laboratories. Pepsin and trypsin after four weeks produced only a small amount of degradation products. Papain acted somewhat more vigorously and was used to prepare a product for testing as an activator.

Ten grams of ball-milled dried activated sludge in 400 ml. of buffer (0.1*M* phosphate) at pH 7.5 after two weeks incubation at 45° to 48° C. yielded a dark, foul smelling liquid on filtration. Evaporation gave 3.0 grams of a dark, odorous, very hygroscopic solid which was evaluated in the usual manner (Table X).

Table X. Evaluation of Papain Hydrolyzate of Dried Activated Sludge on Synthetic Substrate

	Activation, %	
Mg. Added	Papain hydrolyzate	Dried activated sludge
25	...	34.0
50	19.8	34.8
100	34.6	39.0
200	44.3	43.5
300	47.5	46.5
500	51.0	46.7

This material solubilized by the papain showed considerable activity in the fermentation and might be of use as an initial preparation in concentration of the factor.

In summary the fermentation factor seems to be heat and acid stable, alkali labile, and soluble in water, hot ethanol, and 2-propanol. It appears to be absorbed on activated carbon.

Relation of Vitamin B_{12} to Fermentation Factor. At the time we were engaged in these researches the announcement of the isolation of crystalline vitamin B_{12} appeared (*5, 16, 22*). Because our work with synthetic media had been done with substrates containing no vitamin B_{12} and because we soon established the fact that dried activated sludge contained substantial quantities of the new vitamin, it became imperative that we determine what, if any, relationship existed between the fermentation factor and vitamin B_{12}.

Experiments were run, therefore, in which the effectiveness of dried activated sludge and of an aqueous extract thereof was measured in the presence and absence of vitamin B_{12}. Specifically, two synthetic media were prepared as described earlier, these media being identical except that to one we added vitamin B_{12} at the level of 2 γ per 100 ml.

In experiments using the small scale procedure, the addition of the sludge products caused substantially the same increase in fermentation rate in both media. If their vitamin B_{12} contents had been the cause of the activity observed in earlier experiments, then little or no activation should have resulted in the medium already containing vitamin B_{12}. The fact that the usual activity was observed indicates that the fermentation factor is not vitamin B_{12}.

Presence of the Fermentation Factor in Other Materials. During the course of our investigation with dried activated sludge, several materials were found which possessed fermentation factor activity. These materials are: (1) sedimented digested sludge, (2) digested activated sludge, (3) sheep manure, cow manure, (4) Liquafish—a brand of fish solubles containing 50% solids, and (5) Reticulogen—a liver extract.

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