XXVIII. THE ENZYMES OF WASHED ZYMIN AND DRIED YEAST (LEBEDEFF). III. PEROXYDASE, CATALASE, INVERTASE AND MALTASE.

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PEROXYDASE.

On the assumption that hydrogen peroxide and peroxydase might possibly be capable of oxidising some degradation product of sugar, Bach [1906] submitted sugar to the simultaneous action of hydrogen peroxide, peroxydase and an enzyme capable of decomposing sugar. He chose for his experiments the fermenting enzyme of yeast and employed zymine. Being however of the opinion that yeast contains no peroxydase he made use in his investigations of a peroxydase prepared from horse-radish. Although unable to observe any of the oxidation processes the probability of which he had anticipated, he found that when the peroxydase used was previously boiled the amount of carbon dioxide evolved was invariably more than when it was not boiled. This led him to conclude that the presence of active peroxydase had a deterrent effect on the fermenting power of zymine.

The authors find that fresh English brewery yeast contains active peroxydase. When however the yeast is dried for 17 hours at a temperature of 37° it no longer gives the reaction. On washing the dried yeast however the presence of peroxydase can again be detected.

Dried Munich yeast (Schroder) behaves like dried English yeast and does not react for peroxydase when unwashed, but regains that activity on being washed. Whether the peroxydase in yeast has a deterrent effect on the fermentation the authors are at present not prepared to say. It is to be observed that Bach in his experiments used a peroxydase from an outside source (horse-radish) and the presence of an agent in that preparation that would have a retarding effect on fermentation and which could be destroyed by boiling is not impossible.

EXPERIMENTAL.

Experiment 1. Ten g. of pressed English brewery yeast were washed four times to remove adhering wort, etc., and suspended in 300 cc. of distilled water. The washing in this and all the other experiments was done by stirring up the yeast in pots with 120 cc. of distilled water for the first time and 100 cc. for all subsequent times and centrifuging. Into two small Erlenmeyer flasks were introduced:

Flask 1. 10 cc. of yeast suspension (equivalent to 0.1 g. of dried yeast) + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2 (10 per cent. dilution of 3 per cent. H_2O_2 neutralised to litmus).

Flask 2. 10 cc. of yeast suspension + 1 cc. 1 per cent. p-phenylenediamine.

Flask No. 1 showed the first sign of a colouration in one minute and assumed an intense violet colouration in three minutes.

Flask No. 2 remained unchanged.

It is evident from this experiment that English yeast contains an active peroxydase.

Experiment 2. A sample of the yeast used in the previous experiment was then dried for 17 hours at a temperature of 37° and one gram of the dried yeast suspended in 100 cc. of distilled water. Into two flasks were introduced:

Flask No. 1. 10 cc. of yeast suspension (0.1 g. of dried yeast) + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2 (10 per cent. dilution of 3 per cent. H_2O_2).

Flask No. 2. 10 cc. of yeast suspension + 1 cc. 1 per cent. p-phenylenediamine.

Although flask No. 1 after one minute assumed a slightly darker colour than the control flask No. 2, it did not show any violet colouration and the reaction was decidedly negative. The repetition of the above experiments gave similar results and we may conclude that drying inhibits the activity of the peroxydase in the yeast.

Experiment 3. Some dried English yeast was washed four times; this process removes soluble phosphates, the coenzyme of zymase and, doubtless, other substances. Into two flasks were introduced:

Flask No. 1. 10 cc. of washed yeast suspension (0.1 g. of dried yeast) + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. dilute H_2O_2 .

Flask No. 2. 10 cc. of unwashed dried English yeast suspension (0.1 g. of dried yeast) + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. dilute H_2O_2 .

Flask No. 1 showed a colouration after 2 minutes. Flask No. 2 showed no colouration after 5 minutes. This experiment shows that the activity of the dried yeast can be restored by washing.

Experiment 4. 5 g. of dried English yeast were washed four times. The first washings amounted to 32 cc. Six Erlenmeyer flasks were taken and in each were placed 10 cc. of washed yeast suspension (0.1 g. of dried yeast) + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. dilute H_2O_2 .

To flask No. 1 nothing was added.

„ No. 2 0.6 cc. of the washings was added.

„ No. 3 1 cc. „ „ „

„ No. 4 1.5 cc. „ „ were added.

„ No. 5 5 cc. „ „ „

„ No. 6 5 cc. of the neutralised washings were added.

No. 1 showed a colouration after one minute, No. 2, No. 3 and No. 4 after 3, 4 and 4 minutes respectively. No. 5 and No. 6 showed no colouration after 15 minutes. This experiment shows that the addition of the washings inhibits the action of the peroxydase in yeast.

Experiments similar to Nos. 3 and 4 were carried out with dried Munich yeast (Schroder) and zymin, and the results obtained were identical with those obtained with English dried yeast. It is however to be observed that various samples of zymin gave reactions of different intensity.

It is evident from these results that after the yeast cell has been dried or treated with acetone washing removes some substance which has an inhibiting effect on the peroxydase of the yeast. The washings not only inhibit the action of the peroxydase in yeast but also exert a deterrent effect on that of milk as shown by Experiment 5.

Experiment 5. Into two flasks were introduced:

Flask No. 1. 1 cc. dilute H_2O_2 + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. of milk + 5 cc. of water.

Flask No. 2. 1 cc. dilute H_2O_2 + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. of milk + 3 cc. of water + 2 cc. of washings from dried Munich yeast.

Flask No. 1 showed a colouration immediately. Flask No. 2 showed no colouration after 5 minutes.

It was further observed that the action of the inhibiting agent seems to increase in a higher proportion than that of the peroxydase as the amount of yeast is increased.

The following experiment illustrates this point.

Experiment 6. Flask No. 1. 0.05 g. of dried English yeast suspended in 10 cc. of H_2O + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2 .

Flask No. 2. 0.2 g. of dried English yeast suspended in 10 cc. of water + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2 .

Flask No. 1 showed the first signs of a colouration only after 4 minutes and eventually assumed a violet colouration which, although not so intense as in the case of washed yeast, was well marked. Flask No. 2 showed no colouration after 15 minutes.

Several other substances have been found, the addition of which to washed dried Munich yeast (Schroder) inhibits the action of the peroxydase. These are beef broth (0.1 cc.), sodium lactate (0.5 cc. of 1 per cent.), peptone (1 cc. of 1 per cent.), alkalis and acids. Beef broth previously treated with H_2O_2 for 20 minutes still has an inhibiting effect. As to the acids and alkalis, small traces of either will inhibit the action of the peroxydase in yeast as shown by the following experiment.

Experiment 7. Flask No. 1. 10 cc. of washed dried Munich yeast (Schroder) suspension (0.1 g.) + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2 + 0.5 cc. N/10 KOH.

Flask No. 2. 10 cc. of washed dried suspension (0.1 g.) + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2 + 0.5 cc. N/10 H_2SO_4 .

Flask No. 3 contained 10 cc. of washed dried yeast (Schroder) suspension (0.1 g.) + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2 .

No. 3 showed a colouration after two minutes. No. 1 and No. 2 showed no colouration after 5 minutes.

As the washings of dried yeast are slightly acid to litmus the possibility that the acidity of the washings acted as the inhibiting agent on the peroxydase suggested itself, but it was experimentally proved that this was not the case.

Experiment 8. In this experiment the washings were carefully neutralised to litmus.

Flask No. 1. 10 cc. of washed dried Munich yeast suspension + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2 + 3 cc. H_2O .

Flask No. 2. 10 cc. of washed dried Munich yeast suspension + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2 + 1.5 cc. of neutralised washings + 1.5 cc. H_2O .

Flask No. 3. 10 cc. of washed dried Munich yeast suspension + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2 + 3 cc. neutralised washings.

No. 1 showed a colouration after 2 minutes while No. 2 and No. 3 showed no colouration after 5 minutes.

Precisely the same result was obtained when the washings were made neutral to phenolphthalein.

Washed and unwashed Munich dried yeast, washed and unwashed English dried yeast, washed and unwashed fresh English brewery yeast give no reaction with p-phenylenediamine and with benzidine in absence of H_2O_2 , which proves the absence of an oxydase. Similarly the above-mentioned yeasts under the same conditions give no reaction for tyrosinase.

CATALASE.

In the experiments of Bach mentioned earlier in this paper this experimenter also observed that the amount of oxygen evolved by the catalase of the zymin was almost equal to the theoretical amount capable of being liberated from the hydrogen peroxide. In order to ascertain with precision whether the washing of dried Munich yeast influenced the catalase, experiments were carried out as follows: 0.1 g. of dried Munich yeast (Schroder) was introduced into a small Erlenmeyer flask connected with an azotometer filled with mercury over which the liberated oxygen could be collected and measured. 25 cc. of hydrogen peroxide were run in from a burette passing through the stopper of the flask, and the volume of the oxygen liberated and the time taken for the liberation of the gas noted. Then a second experiment was carried out in which 25 cc. of the hydrogen peroxide were treated in the same apparatus with excess of acidified KMnO_4 , and the volume of oxygen liberated again measured. The available oxygen of the hydrogen peroxide, i.e. half the amount evolved on treatment with KMnO_4 , was thus ascertained and compared with the volume evolved by the action of the catalase of the yeast. The washed dried Munich yeast was treated in a similar way.

Experiment 1. 0.1 g. of unwashed dried Munich yeast liberated from 25 cc. of H_2O_2 17.1 cc. of oxygen in 5 minutes.

Acidified KMnO_4 and 25 cc. of the same H_2O_2 liberated 33.6 cc. of oxygen.

Experiment 2. 0.1 g. of washed dried Munich yeast liberated from 25 cc. of another sample of H_2O_2 11 cc. of oxygen in 5 minutes.

Acidified KMnO_4 and 25 cc. of the same H_2O_2 gave 21.9 cc. of oxygen.

It is evident from these results that the catalase in the dried Munich yeast is capable of liberating the entire available oxygen of the H_2O_2 and that the washing of the yeast makes no difference either to the volume of the oxygen liberated or to the rate of evolution.

INVERTASE AND MALTASE.

Experiments were next made to ascertain whether by repeated washing at ordinary room temperature it was possible to remove all traces of invertase and maltase from dried Munich yeast (Schroder) and zymine. The investigations were carried out by means of two different sets of experiments. In one set the yeast was washed six times. The first washings were boiled in order to destroy any invertase and maltase they might contain and added to the washed yeast, the mixture being then digested with cane sugar and maltose respectively for 17 hours at 25° and the CO₂ evolved, if any, noted. One flask containing glucose and another containing water in addition to the mixture of washed yeast and boiled washings were also digested at the same time for comparison. In the event of the invertase and maltase being entirely washed out no fermentation of cane sugar or maltose could be expected to take place, whereas if hydrolysis occurred the resulting hexoses would be fermented by the zymase in presence of the coenzyme added in the washings.

In the second set of experiments the cane sugar and the maltose were respectively digested with the washed yeast without the addition of the washings for 3 hours at a temperature of 25°. The suspensions were then filtered and the filtrate examined in order to ascertain whether any hydrolysis had occurred. In this case, no coenzyme being present, no fermentation could take place.

1. INVERTASE.

Experiment 1. The Munich dried yeast used in this experiment was washed six times.

No. 1. 25 cc. (5 g.) of washed Munich yeast + 20 cc. (1 g.) of cane sugar solution + 30 cc. of boiled washings + 0.5 cc. of toluene.

No. 2. 25 cc. (5 g.) of washed Munich yeast + 20 cc. (1 g.) of glucose + 30 cc. of boiled washings + 0.5 cc. of toluene.

No. 3. 25 cc. (5 g.) of washed Munich yeast + 20 cc. H₂O + 30 cc. boiled washings + 0.5 cc. toluene.

The digestion was continued for 17 hours at 25°.

The contents of all three flasks fermented and the following volumes of CO₂ were evolved :

Time	Flask No. 1	Flask No. 2	Flask No. 3
After 30 minutes	8.9 cc.	8.2 cc.	3.6 cc.
„ 60 „	14.8	14.1	5.5
„ 90 „	18.8	17.7	7.2
„ 17 hours	54.8	57.3	16.0

An experiment in which zymin was substituted for the dried Munich yeast washed six times was also carried out and the results obtained were similar. In 17 hours 25.3 cc. of CO_2 were evolved from the cane sugar, 25.2 cc. from the glucose, and 4.9 cc. from the autofermentation of the zymin.

These results show that the rate of evolution and the volume of the CO_2 produced from cane sugar are almost the same as from glucose, and it may therefore be concluded that the amount of invertase left in the dried Munich yeast and the zymin after washing is very appreciable.

The above results are confirmed by the following set of experiments.

Experiment 2. The dried Munich yeast used was washed six times.

Flask No. 1. 25 cc. (5 g.) of washed dried Munich yeast + 20 cc. (1 g.) of cane sugar solution + 0.5 cc. of toluene.

Flask No. 2. 25 cc. (5 g.) of washed dried Munich yeast + 20 cc. of water + 0.5 cc. of toluene. The contents of the two flasks were incubated for 3 hours at 25° and were then filtered, and the ratio

$$\frac{\text{Rotation in 400 mm. tube}}{\text{Reduction (g. glucose per 100 cc.)}}$$

of filtrate No. 1 was determined, the rotation of filtrate No. 2 being reckoned for in calculating the actual ratio.

$$\text{Ratio } \frac{\text{Rotation in 400 mm. tube}}{\text{Reduction (g. glucose per 100 cc.)}} \text{ of filtrate No. 1} = \frac{-0.373}{0.500} = -0.746.$$

$$\text{Ratio } \frac{\text{Rotation in 400 mm. tube}}{\text{Reduction (g. glucose per 100 cc.)}} \text{ of invert sugar} = -0.800.$$

Experiments similar to Experiment 2 were carried out with dried Munich yeast washed with a mixture of water and toluene and with zymin washed both with water only and with water and toluene. The following are the results obtained:

$$\text{With zymin washed with water} \quad \text{Ratio} = \frac{-0.586}{0.736} = -0.796.$$

$$\text{„ „ „ and toluene} \quad \text{Ratio} = \frac{-0.629}{0.760} = -0.827.$$

With dried Munich yeast washed with toluene and water

$$\text{Ratio} = \frac{-0.614}{0.730} = -0.840.$$

All the above results show that the entire cane sugar was inverted during the three hours.

In view of these results it became of interest to ascertain whether there was any difference in the rate of inversion of cane sugar between washed and unwashed zymin. Zymin was chosen because small quantities of that preparation in a concentrated solution of sugar do not commence fermenting for

some time and consequently it is possible to ascertain the amount of sugar inverted during that time and compare it with the amount inverted in the same time by an equal weight of zymin after being washed.

Experiment 3. The mixtures were incubated for 20 minutes at a temperature of 25°.

Flask No. 1. 0.1 g. of unwashed zymin suspended in 20 cc. of water + 50 cc. of 10 per cent. cane sugar solution.

Flask No. 2. 0.1 g. of washed zymin suspended in 20 cc. of water + 50 cc. of 10 per cent. cane sugar solution.

No fermentation was observed during the twenty minutes. The flasks were then placed in boiling water for twenty minutes in order to stop any further action of the invertase. After cooling to the room temperature they were made up to 100 cc. and filtered and the reducing powers of the filtrates calculated in terms of glucose were determined.

Reduction of filtrate No. 1 = 1.04 g. of glucose per 100 cc.

„ „ No. 2 = 0.67 g. „ „ „

It is thus seen that about $\frac{1}{5}$ of the total cane sugar was inverted by the unwashed zymin during the twenty minutes and that 64.4 per cent. of the amount inverted by the unwashed zymin was inverted by the washed preparation. This would suggest that the amount of invertase left behind even after six washings is a very considerable fraction (approximately 65 per cent.) of that originally present.

2. MALTASE.

A series of experiments similar to those carried out with cane sugar were made with maltose.

Washed dried Munich yeast (Schroder) and washed zymin readily fermented maltose on addition of the boiled washings. The volumes of CO₂ were as follows:

	Maltose	Glucose	Autofermentation
Munich dried yeast	58.0 cc.	57.3 cc.	16.0 cc.
Zymin	25.1	25.2	4.9

As from cane sugar the rate of evolution and volume of the CO₂ evolved from maltose are almost equal to those given by glucose, and consequently the amount of maltase left behind in the preparations after washing must also be very considerable.

The resulting ratios $\frac{\text{Rotation in 400 mm. tube}}{\text{Reduction (g. glucose per 100 cc.)}}$ of filtrates obtained after digesting maltose without the addition of the washings for 3 hours at 25° with dried Munich yeast (Schroder) and zymin respectively, each of these preparations washed with water alone and with a mixture of water and toluene, were :

$$\text{Dried Munich yeast washed with water only} \quad \frac{+ 1.095}{0.550} = + 1.99.$$

$$\text{,, ,, ,, ,, and toluene} \quad \frac{+ 1.848}{0.680} = + 2.7.$$

$$\text{Zymin washed with water only} \quad \frac{+ 2.360}{0.574} = + 4.1.$$

$$\text{,, ,, ,, ,, and toluene} \quad \frac{+ 2.886}{0.490} = + 5.89.$$

Ratio for glucose + 2.1. Ratio for maltose + 9.8.

It appears from these figures that the whole of the maltose was converted into glucose by the dried Munich yeast while only a part of the maltose was converted by the zymin. Washing with a mixture of water and toluene seems however to have made no great difference to either preparation.

The idea of the possibility of removing the maltase from the zymin by a more thorough process of washing suggested itself and an experiment was accordingly instituted in which the zymin was washed 8 times. Each time it was vigorously shaken up in the pot for several minutes and the last time it was placed in a shaker for an hour previous to being centrifuged. On addition of the boiled washings the mixture fermented maltose freely at a rate almost equal to that produced with glucose. It is therefore evident that the most thorough method of washing will not entirely remove the maltase from zymin at ordinary room temperature.

As in Experiment 3 with cane sugar, 50 cc. of 10 per cent. maltose were also digested with 0.1 g. of washed and unwashed zymin respectively for 20 minutes. The resulting filtrates obtained showed the following reductions per 100 cc. calculated in terms of glucose :

Washed zymin 2.2 per cent. Unwashed zymin 2.2 per cent.

In this case although only a part of the maltose was converted into glucose the amounts hydrolysed were equal in both cases, and therefore the quantity of maltase removed by the washing can only have been very small.

SUMMARY.

1. Fresh English yeast reacts with H_2O_2 and p-phenylenediamine for peroxydase.

2. Dried yeast (both English and Munich) does not react for peroxydase with p-phenylenediamine and H_2O_2 but on being washed it regains that activity.

3. The addition of the washings and some other reagents to washed dried yeast inhibits the action of peroxydase.

4. Washing does not affect the activity of the catalase of dried yeast (Schroder).

5. The power of hydrolysing cane sugar is partially but not entirely removed from zymase and dried Munich yeast (Schroder) by washing at ordinary room temperature whilst the power of hydrolysing maltose is not affected.

REFERENCE.

Bach, A. (1906), *Ber.* 39, 1664.