The 'Catalase Test', with Special Reference to *Acetobacter* Species

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The first statement on the occurrence of catalase in bacteria seems to have been made by Beijerinck [1901], who pointed out that whereas the lactobacilli do not contain this enzyme *Acetobacter* species show a positive reaction for catalase. Callow [1923] compared 9 anaerobes and 12 aerobes (none of the aerobes were *Acetobacter* species) with respect to catalase content and noted that the anaerobes were catalase negative and the aerobes were catalase positive. Kluyver [1924], discussing the catalase of micro-organisms, concluded provisionally that this enzyme occurs only in those bacteria which use free oxygen in their metabolic processes. Further information on catalase in bacteria was provided by McLeod & Gordon [1923] and by Virtanen & Karström [1925]. Morgulis, Beber & Rabkin [1926], in their quantitative studies of catalase activity, observed that the activity, which was greatest at 0–10°, diminished with increasing experimental temperatures. The same authors also found that exposure of catalase to temperatures up to 40° for 1 hr. caused practically no inactivation, and cooling the enzyme did not reduce its activity until –10° was reached. The rate of destruction of catalase increased not only with temperature but also with concentration of H_2O_2 , and the destruction followed the course of a monomolecular reaction. Experiments with various H_2O_2 concentrations at different temperatures showed that the maximum activity invariably occurred with 0.54 N H_2O_2 . Using catalase from beef kidney they found its activity to be strong over the range pH 5–9 with greatest activity between pH 6 and 8. The activity was entirely lost at pH 2.

Since Beijerinck's early discovery of the presence of catalase in *Acetobacter* species there have been no further reports on the catalase activity of these particular organisms apart from those of Visser't Hooft [1925, 1939], who recorded the presence of this enzyme in *A. aceti*, *A. rancens*, *A. xylinum*, *A. melanogenum*, and *A. suboxydans*, and who described *A. peroxydans*, a species devoid of catalase. No other catalase-negative acetic-acid bacteria have since been reported.

In recent work with 11 species of *Acetobacter* we observed that very erratic results were obtained when cultures were subjected to the 'catalase test'. When well-developed colonies which had been growing at 25° on malt-extract agar (pH 5.0) in

Petri dishes for 14 days were treated with 0.5 N H_2O_2 , three species gave no response and some of the others reacted only weakly. The results obtained with cultures 8 days' old under conditions which otherwise were identical were practically the same (Table 1).

Table 1

Species of <i>Acetobacter</i>	Reaction with H_2O_2 given by cultures aged		pH developed by the several species when grown in malt extract (initial pH 5.0) at 25° for	
	8 days	14 days	3 days	7 days
(1) <i>A. aceti</i>	—	—	3.85	3.60
(2) <i>A. acetosum</i>	+	+	3.60	3.25
(3) <i>A. kuetzingianum</i>	+	++	4.60	4.30
(4) <i>A. pasteurianum</i>	++	++	4.55	4.05
(5) <i>A. suboxydans</i>	—?	—	4.25	3.70
(6) <i>A. xylinum</i>	+++	+++	4.15	3.50
(7) <i>A. gluconicum</i>	+	+	4.20	3.75
(8) <i>A. ascendens</i>	++	++	4.60	3.90
(9) <i>A. capsulatum</i>	+	—	3.05	2.70
(10) <i>A. acetigenum</i>	++	++	3.80	3.35
(11) <i>A. turbidans</i>	++	++	3.50	3.45

The particular degree of catalase activity shown by each of the several species is probably the resultant of two factors, namely, the quantity of catalase made available by the cells, and the hydrogen-ion concentration produced in the medium by the activities of the cells. Thus, for example, in the cases of the species numbered (4), (6) and (10), which were strong growers and proliferated freely on the slopes, it is probable that the total catalase available was relatively great in amount, hence the influence of the moderate fall in pH value was not so obvious and the cultures reacted in a definitely catalase-positive manner. In the case of the species numbered (1) and (5) where growth was relatively scanty, the moderate fall in pH had a much greater effect and the cultures proved catalase-negative. Finally, in the case of species number (9), although growth was very abundant it was accompanied by a very considerable fall in pH value and the catalase test gave first a doubtful and later a negative result.

The different species were now inoculated into tubes of sterile malt extract (sp.gr. 1.036) buffered with 3% of $CaCO_3$ which had brought the pH to 6.01. After 3 days at 25°, growth was evident in all the tubes, and at this stage in every case the pH value

lay between the limits 5.56 and 5.97. Hence such a medium is a satisfactory one for the control of the acidity developed by *Acetobacter* species. Slopes were then prepared from similar buffered malt extract to which 1.5% of agar had been added. Before addition to the extract the CaCO_3 had been sterilized in steam and afterwards heated at 160° for 2 hr. to ensure that resistant spore-forming organisms were killed. Cultures grown on these slopes at 25° were all found to give a positive catalase reaction on the 3rd day after inoculation and all still reacted positively on the 14th day.

The following experiment was then undertaken. Malt extract agar, buffered with CaCO_3 , was poured in separate 10 ml. portions into a set of conical flasks (250 ml.) and sterilized in the usual manner. These flasks were inoculated with the several *Acetobacter* species. In each case a malt-extract culture grown for 3 days at 20° provided the inoculum, 1 ml. quantities being run from sterile pipettes into the several flasks and spread over the surface of the agar by suitable manipulation of each flask. These flask cultures were incubated at 20° for exactly 3 days and estimations of catalase activity were then performed. Into each flask, including an uninoculated 'blank', there were poured exactly 50 ml. of 0.5N H_2O_2 (adjusted to pH 6.0) and estimations of H_2O_2 were made immediately, then after 15 min., 1 hr., 2 hr., and $3\frac{1}{2}$ hr. These were done volumetrically by the method given by Kolthoff & Sandell [1937]. In the case of the 'blank' the loss (about 10%) of H_2O_2 which was observed up to 45 min. was probably due not to decomposition but to adsorption, but in all the culture flasks much greater losses of H_2O_2 were found to have occurred (Table 2).

Table 2

<i>Acetobacter</i> species	% of added H_2O_2 destroyed after min.			
	15	60	120	210
<i>A. aceti</i>	19.0	23.5	25.0	25.0
<i>A. acetosum</i>	26.0	29.5	30.0	31.0
<i>A. kuetzingianum</i>	16.0	20.5	21.0	21.5
<i>A. pasteurianum</i>	10.5	35.0	35.5	37.0
<i>A. suboxydans</i>	16.5	30.5	32.5	32.5
<i>A. xylinum</i>	11.5	14.5	16.5	16.5
<i>A. gluconicum</i>	18.5	28.5	30.0	30.0
<i>A. ascendens</i>	12.5	18.0	20.5	21.5
<i>A. capsulatum</i>	23.5	40.0	59.0	75.0
<i>A. acetigenum</i>	10.0	16.5	19.5	19.5
<i>A. turbidans</i>	26.0	36.0	42.0	45.0
'Blank'	$7\frac{1}{2}$	15	30	45
Uninoculated medium	3.5	5.5	9.0	10.5

Note. A final set of estimations carried out at 20 hr. from the time of addition of H_2O_2 showed that *A. capsulatum* had destroyed 83.0% and *A. turbidans* 47.0% of the added H_2O_2 .

If the percentages of H_2O_2 destroyed are plotted against the time, the relative catalase activities of the organisms which were grown on the buffered medium in the flasks are indicated very clearly and the curves conform well to the shapes of the curves of catalase activity obtained from estimations conducted by Morgulis *et al.* [1926] who used liver catalase. The relatively high degree of catalase activity shown by both *A. capsulatum* and *A. turbidans* is noteworthy; these are two new species both isolated from sour beers and described, respectively, by Shimwell [1936] and by Cosbie, Tošić & Walker [1942]. From this work the following general conclusions can be drawn relating to the catalase activity of *Acetobacter* species: the acid produced by *Acetobacter* species in a medium containing malt extract inactivates catalase. Young cultures which have not passed beyond the early logarithmic phase of growth show a positive catalase reaction even in unbuffered media, because formation of acid does not take place to an appreciable extent until the organisms are well advanced in logarithmic growth. (An exception is *A. peroxydans* which, as mentioned earlier, is catalase-negative.) Older cultures should not be examined for catalase activity unless grown in a buffered medium similar to that described in this paper. The test should be performed as soon as growth has become well established.

Before examining, for catalase activity, new organisms other than *Acetobacter* species, it would seem desirable, in view of our observations, first to determine whether the organisms in question form acid in the medium used and, if they do so, then the cultures prepared for the catalase test should be cultivated in the presence of a suitable buffering agent. It is also necessary to bear in mind that the rate of loss of catalase activity increases with rise in temperature. Hence the cultures prepared for the test should be incubated at the lowest point of the optimum temperature range for that particular micro-organism.

Finally, it may be remarked that the inactivation of catalase by acid as described in this communication is an interesting parallel to the observation of Butlin [1938] that *A. suboxydans* contains as one of its enzyme components an acid-sensitive system which catalyses a reaction in which the O_2 absorbed is equivalent to the CO_2 evolved. In certain conditions this organism cannot exercise this more highly oxidizing function because of the conditions it creates in the cultures if they are unbuffered (e.g. primary production of gluconic acid can occur in a glucose medium of pH 6.0 by the agency of another oxidizing enzyme present in *A. suboxydans* and this gluconic acid inhibits the first-mentioned oxidizing system).

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Riboflavin, Vitamin B₆ and Filtrate Factors in Wheaten Flours and Offals

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In recent years there have been many studies of the vitamin B₁ content of wheaten flours and breads, but relatively few estimations have been made of the content of members of the vitamin B₂ complex. Some five years ago Copping & Roscoe [1937] published a survey of the water-soluble B vitamins in yeast, flour and bread, and gave values for the riboflavin and 'factors other than flavin'. These estimations were, however, made at a time when our knowledge of the members of the vitamin B₂ complex was more incomplete than it is at present, and the methods used appear far from satisfactory in the light of later work. New methods for the biological estimation of riboflavin have been described by El Sadr, Macrae & Work [1940], and on the basis of these methods others have now been evolved for estimating vitamin B₆ and filtrate factors. Almost all of the previous values for riboflavin and vitamin B₆ which have been put forward for flours have been obtained by chemical or microbiological methods.

The term filtrate factors is taken to mean all growth factors of the vitamin B₂ complex required by the rat, other than riboflavin, nicotinic acid and vitamin B₆ [El Sadr, Hind, Macrae, Work, Lythgoe & Todd, 1939; Macrae, Todd, Lythgoe, Work, Hind & El Sadr, 1939]. Filtrate factors are now known to include pantothenic acid, biotin and one or more unidentified substances. Lepkovsky [1942] reviews the subject most lucidly.

It is important at the present time to have some precise indication of the nutritive value of the flour and bread available to the people of this country, and on this account, as well as to obtain values by biological methods for comparison with those obtained by other methods, the further study of wheaten flours was undertaken.

MATERIALS

The materials used were supplied through the courtesy of the Research Association of British Flour Millers, who arranged to have suitable flours milled and to store some of the grist so that further samples could be milled if required. In January 1941 the following samples were milled and put into store in a cool place in airtight containers: (1) straight run unbleached white flour of 73% extraction; (2) whole-meal flour of 100% extraction; (3) weatings or middlings; (4) bran; (5) germ. The germ represented all the clean germ obtained in the milling of the white flour, and the bran and weatings together comprised the balance of the wheat after removal of 73% as white flour.

About 6 months later, when the Accessory Food Factors Committee of the Medical Research Council and the Lister Institute had laid down the specification for the preparation of the 85% extraction national wheatmeal [Medical Research Council, 1941], a sample of this type of flour was milled from the original grist and tested in the same way as the other samples.

Table 1. *Composition of the flours and offals (%)*

	Water*	Ash	Nitrogen	Protein
73% flour	13.6	0.411	2.042	11.64†
85% flour	12.64	0.861	2.103	11.98†
100% flour	13.53	1.545	2.190	12.48†
Weatings	14.6	4.31	3.04	17.33†
Bran	13.9	6.55	2.495	15.6†
Germ	13.98	4.18	4.76	29.75†

* Dried at 105–110°.

† N × 5.7.

‡ N × 6.25.