XXXIX. FERMENTATION BY YEAST PREPARATIONS.

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THE striking difference between the fermentation of living yeast and that of the juice prepared from it is well known [Harden and Young, 1906]. A living yeast for example may ferment in an excess of sugar solution at a rate of 10–15 cc. CO₂ per 5 minutes for 2 g. of pressed yeast. The fermentation is unaffected by the addition of phosphate except in high concentrations, when a slight inhibition may occur. The yeast juice prepared from such a yeast ferments at 1/20–1/40 of this rate; the addition of phosphate to the juice produces temporarily a rate of the same order as that of the original yeast, which falls to the normal as the phosphate becomes esterified, but can be maintained indefinitely in a suitable concentration of arsenate [Harden and Young, 1911]. It seemed of interest to determine at what point in the preparation of yeast juice, or by what reagents or treatment, this modified fermentation was produced.

The preparation of yeast juice is usually carried out by grinding fresh pressed yeast with equal parts of silver sand and kieselguhr, and pressing out the ground mass after the addition of more kieselguhr in a hydraulic press. It is apparent that changes may occur either in the preliminary grinding, or by the retention of active material in the residue during pressing, or in both processes, and these were the possibilities first investigated.

Experiments on grinding yeast.

150 g. of fresh pressed brewery top yeast were mixed thoroughly with 150 g. of sand. A portion was removed to determine the original rate of fermentation, and the remaining mass ground in a mill, samples being removed at regular intervals for examination. The fermentations were carried out in the apparatus of Harden, Thompson and Young [1910] at a temperature of 30°. 4 g. of each sample (containing 2 g. yeast) together with 2 g. fructose and 20 cc. H₂O were incubated, the rate of fermentation and the response to phosphate being determined. Fig. 1 illustrates a typical experiment.

It will be seen that the fermentation rate (cc. CO₂ per 5 minutes) diminished approximately in proportion to the duration of grinding; after grinding for

1 hour (curve *c*) the rate had fallen from 13.5 cc. to 2.5 cc., and rose to 10 cc. on addition of phosphate, giving a typical phosphate curve. Microscopic examination of the ground mass at this stage showed that 70 % of the cells were now permeable to methylene blue, but no estimate was possible of the number which had been disintegrated by the grinding. Without addition of phosphate the rate of fermentation of the ground mass increased during 2 hours to double the initial value, corresponding then to the final rate after addition of phosphate; it is possible that this increase may be due to growth of the surviving intact yeast cells.

It appears from these results that the essential change in the mode of fermentation takes place during grinding, and that during pressing only secondary effects which may enhance this change can occur.

Fig. 1. Fermentation of yeast after grinding.
a, 2 g. yeast. *b*, 2 g. yeast ground 20 minutes.
c, 2 g. yeast ground 60 minutes.

↓ marks addition of 1.2 cc. $M K_2HPO_4$.

Experiments on grinding and pressing with various substances.

It was thought possible that the presence of the kieselguhr used as packing material during grinding and pressing, which could conceivably act as an adsorbent of active material, might account for the low basal rate of yeast juice. Preparations were therefore carried out in which other materials were substituted for the kieselguhr.

One of the difficulties encountered in the investigation of a series of yeast juices is the unaccountable manner in which juices prepared identically from different batches of fresh yeast from the same source vary both in their basal rate and their power to respond to phosphate. To provide a standard for comparison, it was therefore necessary on each occasion to prepare a juice in the normal way with kieselguhr.

Fresh pressed brewery top yeast was used throughout. The yeast was mixed with sand and the material to be tested, and ground to the usual consistency. After standing for 2 hours the ground mass was pressed out, and the volume and p_H of the juice were determined. Duplicate flasks were

prepared containing fructose and a measured volume of juice and placed in a thermostat at 30°; the basal rate of fermentation and the phosphate rate were then determined. There was on occasion a noticeable difference in p_H between the experimental juice and the parallel kieselguhr juice, and in those cases a portion of the control juice was adjusted to the same p_H , without appreciable difference to the fermentation; the influence of the p_H during the fermentation may therefore be discounted, though it may be of importance in the preliminary pressing.

Table I.

Exp.	Packing material	Yield of juice cc./g. yeast	p_H	Rate cc. CO ₂ per 5 min. for 25 cc. juice	
				Basal	Max. phosphate
60	CaCO ₃	100/500	6.0	8.0	51.0
61	CaCO ₃	65/500	6.2	11.4	65.0
	Kieselguhr	65/500	6.0	7.5	74.0
62	CaCO ₃	148/500	6.1	5.3	63.4
	Kieselguhr	160/500	5.4	1.9	29.0
72	CaCO ₃	180/500	5.6	6.0	49.5
	Kieselguhr	154/500	5.4	1.5	26.0
66	Powdered glass	108/500	7.0	6.4	16.7
	Kieselguhr	170/500	5.6	2.4	16.0

Aluminium oxide, ferric oxide, barium carbonate and sulphate, and calcium phosphate gave juices which, though normal in yield and appearance, were inactive or of very poor activity. Magnesium carbonate gave a normal yield of juice with a basal rate lower than normal, which responded only slightly to phosphate; with aluminium hydroxide an extraordinarily poor yield was obtained, approximately 10 % of the usual volume; the juice however had a basal rate of fermentation twice that of the control but a phosphate rate of the same order as the control. The only substances used which had any beneficial effect on the fermentation rate were calcium carbonate and powdered glass. With calcium carbonate both the yield and the p_H of the juice obtained corresponded fairly closely with the control, but the basal fermentation rate was doubled or trebled. Table I shows some of the values obtained for the juices. Microscopic examination showed that both with the calcium carbonate and the kieselguhr, the number of cells in the ground mass permeable to methylene blue was the same, about 70 %.

To determine whether any active material could be adsorbed by kieselguhr, 50 cc. portions of the juices prepared with CaCO₃ were mixed with 5 g. kieselguhr, allowed to stand at room temperature for 10–30 minutes, and then filtered. On determination of the fermentation rate of the filtrate a slight diminution in the basal rate was observed; on one occasion the rate fell from 11.4 cc. CO₂ for 25 cc. to 9.5 cc. after treatment, and on another, from 5.3 cc. to 3.1 cc.

In the latter case the kieselguhr residue was suspended overnight in

20 cc. 0.1 *M* K_2HPO_4 , and the filtrate tested for the presence of hexose-phosphatase by its hydrolysing action on potassium hexosediphosphate. It was found that 10 cc. filtrate, equivalent to 25 cc. original juice, liberated 0.4 mg. P in 5 minutes, which corresponds to a rate of 0.29 cc. CO_2 , whereas the diminution in rate of the juice was 2.2 cc. Though the elution was far from complete, it demonstrated that active material was adsorbed on the kieselguhr. It was apparent, therefore, that though the main diminution in rate originated in the grinding of the yeast, the quality of the packing material might account for a decrease in rate up to at least 10 % of the total.

Effect of chemical reagents on fermentation by yeast.

The ideal reagent sought after in these experiments was one which, while killing the cell so that it was incapable of growth, would leave the fermentation rate unaffected. This was however not found. The action of some reagents has already been investigated. It was shown by Harden [1911] that the addition of toluene to fermenting yeast causes a rapid fall in the fermentation rate until a constant low rate is obtained, while simultaneously the yeast acquires the property of responding to phosphate. Kerr and Young [1926] showed that benzene, xylene, ether and ethyl butyrate acted in a manner similar to toluene, and that after treatment the yeast cells were incapable of growth; chloroform, ethyl acetate, amyl acetate and butyl alcohol completely stopped the fermentation.

Table II. *Fermentation of yeast in presence of various reagents.*

Reagent	Rates, cc. CO_2 per 5 min.		Reaction to PO_4	% cells stained
	Original	After addition		
Formaldehyde 2 %	44	4.0	—	15–20
Phenol 0.25 %	32	16.0	—	.
„ 0.5 %	30	1.0	—	.
Pyridine 2.2 %	22	7.0	—	.
Propyl alcohol 7.5 %	28	9.0	—	80
Quinone 2.5 %	26	15.0	—	.
„ 1 %	26	0	—	28
Toluene	32	1.5	+	100
Benzene	32	1.5	+	100
Carbon disulphide	28	2.0	+	100
Petroleum 12.5 %	27	3.5	+	100
Light petroleum 6.25 %	30	7.0	+ slow	75

A list of the reagents now investigated is given in Table II. The fermentations were carried out with fresh pressed brewery top yeast. A series of flasks was prepared containing a weighed amount of yeast, fructose and water and placed in a thermostat at 30°. When a steady rate of fermentation was obtained, usually after 10–15 minutes, the reagent to be tested was added to the flasks and the subsequent fermentation measured. After the rate had again become constant, the response of the fermenting mass to added phosphate was determined. A typical experiment is illustrated (Fig. 2).

- (a) 8 g. pressed yeast + 3 g. fructose + 40 cc. H_2O .
 (b) 8 g. „ + 3 g. „ + 40 cc. H_2O + 5 cc. carbon disulphide.
 (c) 8 g. „ + 3 g. „ + 40 cc. H_2O + 4 cc. 4 % formaldehyde.

In Fig. 2 the arrow marks the addition of 2.4 cc. M K_2HPO_4 to b and c , the dotted portion (b') of curve b representing the fermentation in absence of phosphate. In some cases a count was made microscopically of the proportion of cells staining with methylene blue after treatment with the reagent.

It will be seen that the fermentation was effected in one of two ways by the reagents tested. The first group, which includes phenol, pyridine and formaldehyde, reduced and with increasing concentration finally stopped the fermentation. No response to added phosphate occurred, indicating that the fermenting capacity was definitely lowered or destroyed; the cells, however, were not necessarily so injured that they stained with methylene blue. The second group, to which toluene, benzene and xylene were already known to belong, all reduced the fermentation roughly to the same degree, about 1/20 of the normal. The treated yeast then reacted with phosphate to give a definite phosphate rate, approximately 40–60 % that of the original yeast, and this rate could be maintained in the presence of arsenate. Moreover, on staining with methylene blue practically 100 % of the cells were permeable to the dye.

Fig. 2. Effect of reagents on fermentation by yeast.

a , Normal. b , In presence of carbon disulphide.

c , In presence of formaldehyde.

↓ marks addition of K_2HPO_4 .

Assuming that in the living yeast fermentation takes place according to the equations of Harden and Young:

or some similar equations, the enzymes must be so balanced as to produce the optimum concentration of phosphate. In the treated yeast, the limiting factor, as evidenced by the response to phosphate, is the hydrolysis rate of the hexosephosphates produced. The results quoted above lead to the conclusion that injury to the yeast cell, of which grinding is the extreme case and drying, and treatment with acetone, toluene etc. are intermediate gradations, results not in any significant diminution of fermenting capacity but in an inability to maintain a high rate and is confined to some special mechanism

or organisation existing in living yeast which permits a more intense action of the hexosephosphatase.

The possibility therefore exists that by suitable treatment a substance could be extracted from yeast which would accelerate the action of hexosephosphatase and so increase the basal rate of yeast preparations, *e.g.* yeast juice or zymin, in a manner similar to arsenate.

Search for an accelerator of hexosephosphatase in yeast.

All attempts to prepare such an extract from living yeast, as by autolysis or plasmolysis, rubbing in a mortar etc. failed completely. Autolysis of a dried baker's (Dutch) yeast, which is a very active preparation fermenting at a rate 40 % that of living yeast, gave an extract accelerating the fermentation of zymin. 100 g. of dried yeast with 200 cc. H_2O were incubated for 16 hours at a temperature of 30° and the clear brown liquid separated by filtration. The accelerating power was measured in the following manner. Duplicate flasks were prepared containing 2 g. zymin, 2 g. fructose, 2.4 cc. $M K_2HPO_4$ and 10 cc. H_2O , and placed in a thermostat at 30° till a constant rate of fermentation was obtained. Preliminary experiment showed that this quantity of phosphate was sufficient to give the optimum concentration of phosphoric esters necessary for the maximum basal rate [see Boyland, 1929] and therefore no error would be incurred by the subsequent addition of extracts containing phosphate. A known volume of the autolysate was added to one flask and an equal volume of H_2O to a control flask, and the fermentation rate measured. In one experiment, the rate of fermentation was raised from 3.3 to 6.9 cc. CO_2 per 5 minutes, and in another from 3.85 to 8.05 cc. by the addition of 15 cc. autolysate. The acceleration was not produced if the autolysate was boiled previously to addition. Further examination of the autolysate showed that it contained an active hexosephosphatase. The hydrolysing power for potassium hexosediphosphate was measured under standard conditions at p_H 7.0, the inorganic phosphate liberated being estimated by a modification of Briggs's method. In one case 15 cc. autolysate, which produced an acceleration of 4.20 cc. CO_2 per 5 minutes in the rate of fermentation of zymin, hydrolysed 57.0 mg. P in 2 hours, corresponding to a rate of 1.8 cc. CO_2 per 5 minutes and thus accounting for 43 % of the acceleration. The figures obtained cannot fairly be compared, however, as the conditions existing during fermentation and hydrolysis are necessarily very different.

Attempts were made to separate the hexosephosphatase from any accelerating agent which might be present by means of precipitation with alcohol or saturated ammonium sulphate and by dialysis, but without success. From this point of attack, it remains impossible at the moment to decide whether the added hexosephosphatase alone is responsible for the acceleration, or whether it is accompanied by an accelerating system with which it is closely associated.

SUMMARY.

1. Investigation of the manner of preparation of yeast juice shows that at least 80 % of the diminution in the rate of fermentation which occurs may be ascribed to the process of grinding, during which the yeast acquires the power of responding to phosphate. A further loss is incurred during pressing by the use of kieselguhr, which adsorbs active material.

2. The effect of various reagents on the fermenting power of yeast is described.

3. The conclusion is drawn that the treatments described affect mainly the mechanism of hexosephosphatase action.

4. An account is given of the preparation, by autolysis of dried baker's yeast, of an extract containing hexosephosphatase and accelerating the fermentation rate of zymine.

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