CI. DIRECT FERMENTATION OF MALTOSE¹.

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(Received December 18th, 1933.)

WILLSTÄTTER designated as "direct fermentation" of disaccharides the fermentation of sugars like sucrose, maltose or lactose without preceding hydrolysis into monosaccharides. The assumption of such direct fermentations conflicts with the prevailing hypothesis that any glycolysis of polysaccharides must be preceded by cleavage of the glucosidic linkages. This classical hypothesis, perhaps first formulated by Emil Fischer and Lintner, has been generally accepted, as it seems to be supported by ample experimental evidence, such as the correlation of fermentability and non-fermentability of carbohydrates by yeasts and other micro-organisms with the presence or absence of specific carbohydrases.

The validity of this theory was questioned by Willstätter and Steibelt [1921]. They found that brewer's yeast generally contained ample maltase to accomplish the conversion of maltose in the wort into glucose for subsequent fermentation. Table I gives a synopsis of the time required for half fermentation and for half hydrolysis of maltose under comparable standard conditions for various yeasts studied in this laboratory and including some examples given by Willstätter and Steibelt [1921] and Willstätter and Bamann [1926, 1]. Munich brewer's yeast (Löwenbräu) ferments maltose rapidly, but can hydrolyse it still faster. With various distiller's yeasts, the time of half hydrolysis is of the same order of magnitude as that of half fermentation, but the maltase content seems to be less than sufficient. A third group, comprising yeast M of the Berlin Institut für Gärungsgewerbe, Vienna distiller's yeast, and New York Lager yeast, is distinguished by a paradoxical lack of maltase. The amount of maltase found is negligible. Although the determination of maltase in such small amounts is not very accurate, even the lowest values for half hydrolysis (highest maltase values) are many times larger than the time required for half fermentation of the same quantity of maltose. The maltase content is far from sufficient to supply the zymase with those amounts of glucose necessary, according to the theory, for the maintenance of the observed swift development of carbon dioxide and alcohol. Willstätter and Oppenheimer [1921-22] observed an analogous discrepancy between the fermentation of lactose and the low amount of lactase in a strain of *Saccharomyces fragilis* Jørgensen.

Before discussing various attempts to reconcile these facts with the classical theory, one might define "direct fermentation" in the light of the present knowledge regarding the zymase complex. The absence of disaccharidase, *e.g.* maltase, as shown by the absence of glucose from the fermenting mixture, leads to the assumption that the sequence of intermediaries in disaccharide fermentation converges at a later stage with that of common hexose fermentation. Thus, we may suspect the existence of disaccharide-phosphates and possibly the transi-

¹ Read before the Section of Biological Chemistry at the Semiannual Meeting of the A. Chem. Soc., Chicago, September 1933.

tory formation of C_6-O-C_3 or C_3-O-C_3 derivatives. The eventual cleavage of the glucosidic linkage in any such molecule could hardly be considered as "maltase" action. The further course of direct fermentation is indicated by the dotted arrows in the diagram.

In order to preserve the classical conception, it may be argued, (1) that the competitive inhibition by glucose, the product of hydrolysis, impairs maltase action in the maltase determination, in contrast to conditions during fermentation when the product of hydrolysis is constantly removed; (2) that the hydrolytic enzyme is regenerated during fermentation; (3) that in these paradoxical cases the method of maltase determination fails to give the full value of maltase activity. The maltase might be present, but not in an extractable form; the active group of the maltase might even be linked with the same colloidal carrier as that component of the zymase system which initiates glucose fermentation, and the glucose molecule would be handed over by a conveyor-like mechanism from the maltase to the zymase without the possibility of glucose being detected or isolated. While such a mechanism would still fall under the definition of direct fermentation, positive proof for it could only be adduced again by the separation of such a "malto-zymase" into maltase and zymase.

Point (1) can easily be refuted since the affinity of glucose for maltase is quantitatively known; thus, the inhibitory effect of the monosaccharide on the enzyme can be evaluated and shown to be of no consequence in comparison with the observed divergencies. Point (2) was raised by Euler and Brunius [1926], when Willstätter and Lowry [1925], and Willstätter and Bamann [1926, 1] succeeded in demonstrating direct fermentation of sucrose and of maltose with yeasts depleted of the respective disaccharidases by treatment with mineral acid or alkali hydroxide. Sucrase determination after fermentation sometimes showed increments, but not sufficient to account for the rate of fermentation. In some instances, *e.g.* in the case of a lactose-fermenting yeast, the enzyme content after fermentation was just as low as before fermentation. No galactosazone, but only lactosazone, could be isolated from a partially fermented lactose solution. A further contribution by Willstätter and Bamann [1926, 2] showed that even yeasts rich in maltase will ferment without antecedent hydrolysis at p_H 5, at which maltase is practically inactive whilst fermentation proceeds at an optimum rate.

The authors feel that this problem forms a part of the general question, whether the metabolism of high-molecular compounds follows exclusively those patterns presented by the action of non-specific laboratory reagents such as acids and alkalis. The following report includes unpublished observations on yeast M made by one of us in 1921 under the direction of Prof. R. Willstätter [Sobotka,

1922], and a series of experiments with a peculiar yeast which was very kindly supplied by Mr Leo Wallerstein.

Yeast M of the Berliner Institut für Gärungsgewerbe, recommended for the fermentation of molasses, is, according to German authors, a mixture of five strains of yeast, among them yeasts II and XII. This statement is hardly in agreement with the fact that yeasts II and XII have maltase values of 1.3 and 4–6, while no sample of yeast M ever contained more than 0.4 maltase units per g. Samples of yeast M of maltase values 0.1–0.05 fermented maltose almost as fast as glucose, except, under the chosen conditions, for a peculiar period of induction of about two hours. Such prolonged inductions have never been observed with live yeast, whilst they are familiar with dry yeast and yeast juice fermentations [Sobotka, 1924]. Contact of the yeast with maltose solution abolishes induction, but even a prolonged stay of the yeast in a maltose medium and frequent renewal of maltose failed to enhance the maltase content of the yeast, while its fermenting power for maltose reached that for glucose during this maltose treatment.

The possibility of maltase formation during maltose fermentation was excluded in the following experiment. A maceration juice prepared according to Lebedev [1912] from an air-dried brewer's yeast, rich in maltase, contained exactly the same amount of maltase as the fresh yeast from which it was derived. A maceration juice from dry yeast M corresponded in its poor maltase content with fresh yeast M, whilst it fermented maltose. This observation supports the assumption of direct fermentation, as it excludes regeneration of maltase during fermentation, which is inconceivable in the cell-free juice. It also corroborates the soundness of the maltase determination with ethyl acetate on fresh yeast, as any cell-bound enzyme, which might have escaped that determination, should have been liberated during maceration.

Proof that the "malto-zymase" is not only free from a maltase component but actually follows a different initial path from "gluco-zymase" was obtained with the yeast from the Wallerstein Laboratories. This strain is distinguished by its ability to live, to ferment and to propagate at the rather acid p_H 2.5. This yeast is free from maltase both at p_H 2.5 and at p_H 6.5, the optimum for the action of yeast-maltase. None the less, it speedily ferments maltose or malt extract media, and, in several experiments, the rate of maltose fermentation exceeded that of glucose fermentation. These observations can be interpreted only on the assumption of direct fermentation of maltose.

EXPERIMENTAL.

Determination of maltase value and maltase units. Willstätter *et al.* [1924] defined as Maltase Unit (M.U.) that amount of enzyme which hydrolyses 2.5 g. maltose hydrate in 50 ml. of a phosphate buffer solution of p_H 6.8 at 30° to the extent of 50 % within one minute. A yeast has the Maltase Value (M.V.) = 1 if it contains 1 M.U. in 1 kg. dry substance.

The M.V. is usually estimated in the following manner. After the dry weight of the yeast has been determined, an amount equivalent to 2.5 g. dry yeast, usually about 10 g. fresh yeast, is liquefied with 1 ml. ethyl acetate, following the procedure of Willstätter and Steibelt [1920]. After cautious neutralisation with a suitable amount of $N/10$ ammonia, the solution is made up to 50 ml.; 40 ml. of this are added to 50 ml. of a 10 % maltose hydrate solution *plus* 5 ml. of $M/3$ phosphate buffer (equal parts of $M/3$ KH_2PO_4 and Na_2HPO_4) and made up to 100 ml. Samples of 25 ml. are withdrawn at appropriate intervals, *e.g.* 10, 20 and 40 minutes, and the action of the enzyme is interrupted by running the sample

into 5 ml. of a 2*N* Na₂CO₃ solution, which at the same time catalyses complete mutarotation of the products of hydrolysis [Hudson, 1908]. The samples are clarified by filtration, if necessary with addition of charcoal. The rotation in a 2 dm. tube of the clarified solution will be found to be +10·80° for a sample taken immediately after addition of the enzyme, and +4·40° after complete hydrolysis. Thus, percentage hydrolysis can be determined and the time for 50 % hydrolysis can be interpolated by means of the typical reaction curves [see Bamann, 1929].

If 50 % hydrolysis is accomplished, *e.g.* in 70 minutes, 50 ml. of the reaction mixture, equivalent to 1 g. dry yeast, contain 1/70 M.V. Thus, the M.V. of this yeast would be 1000/70 = 14·3.

Fermentation of sugars can be measured in a similar way. 20 ml. of a 5 % sugar solution, saturated with carbon dioxide, are fermented with fresh yeast equivalent to 0·2 g. dry yeast, and the carbon dioxide developed is measured, preferably over mercury. The amount of carbon dioxide developed under 760 mm. Hg pressure and 30° is 282 ml. The time within which half this amount is liberated can be correlated with the time of half hydrolysis under the conditions of the maltase determination, which are all identical except that the double amount of yeast is employed. The values for half fermentation of maltose, given in Table I, were obtained by this procedure.

Table I. *Fermentation and hydrolysis of maltose by various yeasts.*

Yeast	Time of half fermentation mins.	Time of half hydrolysis mins.	Maltase value
Munich brewer's	80	60	33·3
Danish distiller's	255	320	6·2
XII, distiller's	205	300-760	6·6-2·6
New York baker's	600	400-900	5·0-2·2
New York ale	—	900	2·2
Rhine wine	—	500-640	4·0-3·1
New York lager	460	4,000	0·5
Munich brewer's, acid-treated	170	4,000	0·5
Frankenthal distiller's	240	50,000	0·04
Vienna-Stadlau distiller's	1200	8,000-25,000	0·25-0·08
M, distiller's	480	5,200-16,000	0·38-0·12
A, grown at p_H 2·5	800	40,000	0·05

Induction period. Yeasts poor or lacking in maltase ferment maltose, as a rule, only after a delay of 10 minutes to 3 hours under the above conditions. After this induction period which lasts from 2 to 3 hours with yeast M, the development of carbon dioxide soon reaches the linear course typical for alcoholic fermentation. If this period of induction is subtracted from the time of half

Table II. *Fermentation of glucose and maltose by yeast M.*

	Time for half fermentation			Corrected time for half fermentation mins.
	Glucose mins.	Maltose mins.	Induction mins.	
Original sample (1. vii. 21)	200	380	120	260
After glucose passage	270	390	110	280
After 1 maltose passage	250	280	none	—
After 2 maltose passages	240	255	none	—
Same, 5 times washed	205	335	none	—
Original sample (22. vii. 21)	—	390	85	305
After maltose passage	210	220	none	—

fermentation in the case of yeast M, it is found that the maltose fermentation, once it has started, proceeds at the same rate as glucose fermentation. If fresh yeast M is carried through one or two passages in diluted malt extract, the induction period disappears. Maltase determinations in yeast M, after maltose treatment had eliminated the induction period, still yielded values of 9000 and 17,000 minutes as time for half hydrolysis. Analysis of reaction mixtures at the end of the induction period proved the absence of glucose.

Maltase determination in maceration extract. Maceration extract [Lebedev, 1912] was prepared in the following manner. Yeast was dried in a 2 mm. layer between 30° and 40°. One part of dry yeast was incubated at 30° for 4 hours with three parts of water. Three ml. of clear juice, isolated by centrifuging, corresponded to 1 g. dry yeast. Three or six ml. of juice were used in a total volume of 100 ml. buffered solution containing 5 % maltose; fermentation was effectively prevented by the addition of 5 ml. ethyl acetate. The figures given in Table III show that fresh maceration extract from brewer's yeast contains an

Table III. *Maltase determinations in fresh yeasts and maceration extracts.*

$p_H = 6.8, t = 30^\circ$					
	Dry yeast equivalent g./100 ml.	Time min.	Decrease in rotation ($l = 2$ dm.)	% hydrolysis	Time of half hydrolysis, calc. for 1 g. dry yeast in 100 ml. min.
Löwenbräu yeast	2.0	40	2.80°	43.8	56
	—	80	3.72	58.2	56
Löwenbräu, maceration extract, 10 ml.	3.33	40	4.14	64.7	63
Same, after 24 hours, 6 ml.	2.0	30	1.74	27.2	235
	—	65	2.52	39.4	235
	—	120	3.25	50.8	330
Yeast M	2.0	160	0.25	3.9	7000
Same	2.0	450	1.00	15.6	5200
Yeast M, maceration extract, 6 ml.	2.0	1200	1.92	30.0	7800
Same, after 24 hours, 6 ml.	2.0	91	0.10	1.6	14,000
	—	132	0.18	2.9	13,500

amount of maltase identical with that found in the fresh yeast. It deteriorates like other enzymes in maceration extract rather rapidly. On the other hand, a fresh extract made from dried yeast M contained minute amounts of maltase only, of the same order of magnitude as the fresh yeast.

Yeast A. The yeast obtained from Mr Wallerstein, briefly designated yeast A, grows in a 10 % solution of malt extract the p_H of which is brought to 2.5–2.8 by addition of 0.8 volume % concentrated sulphuric acid. No maltase was found in this yeast when it was tested either at p_H 6.5 or at p_H 2.5. However, it fermented both maltose and glucose.

Fermentation was carried out in a Barcroft-Warburg apparatus, both in oxygen and in nitrogen, and the rate of carbon dioxide development was measured. This varied between 100 and 250 mm.³/30 minutes, when an amount of fresh yeast equivalent to 4 mg. dry yeast was allowed to act on 50 mg. sugar in a total volume of 2 ml. *M*/20 primary phosphate solution. A comparison between maltose and glucose fermentation showed no significant difference between the two sugars. However, with one batch, the fermentation of maltose

was definitely faster than that of glucose. 208 mm.³ carbon dioxide were developed from maltose in thirty minutes as compared with 161 mm.³ from glucose under anaerobic conditions; under aerobic conditions the figures were 129 mm.³ for maltose and 73 mm.³ for glucose.

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