88. The Direct Fermentation of Maltose by Yeast. 2*

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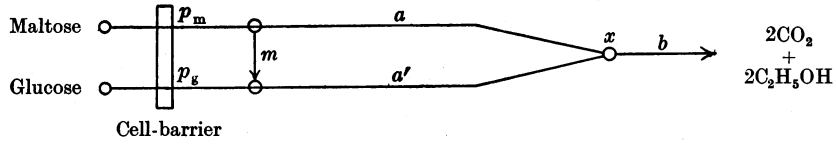
Discussion on the existence of disaccharide-specific zymase systems appears to be entering a new phase. The fermentative breakdown of maltose, sucrose, lactose and trehalose has been investigated in recent years from different directions. Nevertheless, the question whether hydrolysis is an indispensable preliminary to the fermentation of a disaccharide is still variously answered. Supporters of the view that disaccharide fermentation may be 'direct' have as a rule been content, hitherto, with negative proofs showing that oligases are not indispensable to disaccharide fermentation. There is clearly need, however, for a positive hypothesis concerning the mechanism whereby disaccharides could be fermented directly.

Hydrolysis to glucose appears to be one way but not the only way in which maltose is broken down by the yeast cell. Pictorially this view may be represented as follows:



Here p_m and p_g are the respective permeation processes whereby maltose and glucose become available to the action of intracellular enzymes. m is the hydrolytic reaction mediated by maltase and thus the path followed in indirect maltose fermentation. a' and b represent the reaction sequence of glucolysis; a and b represent the reaction sequence of direct maltose fermentation to be referred to below as maltolysis. The unidentified intermediary in which the direct fermentation paths of maltose and glucose presumably merge has been designated as x. Following Willstätter, the enzyme systems responsible for *direct* fermentation of maltose and glucose are termed maltozymase and glucozymase respectively.

Let us term the over-all velocity of maltose fermentation and glucolysis respectively V_m and V_g. Conceivably the ratio V_m/V_g may be equal to, greater than, or less than unity. In considering the bearing of kinetic evidence on the possible existence or non-existence of a direct maltose fermentation, careful distinction between these three cases will be useful.

(1) $V_m/V_g < 1$. Since in this case maltose is fermented more slowly than glucose, the rate-limiting part-reaction of the maltose fermentation cannot be contained in the glucolytic sequence *a' and b*. If a given activator or inhibitor of maltose fermentation, but not of glucose fermentation, is found to be without action at stages p_m and m of the scheme presented, the existence of this specific activator or inhibitor must then be regarded as convincing, if indirect, evidence of the existence in the cell of a specific sequence a of maltolysis.

(2) $V_m/V_g = 1$. Unless evidence is advanced to the contrary, it is plausible to assume in this case that the rate-limiting reaction of maltose and glucose fermentation is the same, i.e. that the rate-limiting reaction is contained in the common component b. As

* Cf. preliminary note [Leibowitz & Hestrin, 1940], earlier investigation [Leibowitz & Hestrin, 1939], and dissertation by Hestrin [1941].

long as b continues to set the pace, the presence of specific activators or inhibitors of maltolysis will not affect the rate of gas liberation.

(3) $V_m/V_g > 1$. The simplest explanation of this case is to assume that a exists and that its velocity is greater than a', the latter being rate limiting for glucolysis but not for maltose fermentation. This interpretation is only necessary, however, under certain conditions. Their specification will not be necessary in the present connexion. As in case (2) the limiting part-reaction of maltose fermentation may be contained in b. While the latter remains limiting, specific activators and inhibitors of maltose fermentation need not necessarily affect the rate of gas liberation.

Conditions as specified under (1) were found in baker's yeast; conditions as specified under (2) and (3) in a brewer's yeast.

Methods

Yeast. Except where otherwise stated, the yeast used for tests was a Palestinian brand of commercial baker's yeast supplied by Paca Ltd. Samples purchased at different dates were left to stand on ice for a number of days before use. This treatment lessened the fermentative power of the yeast to maltose, augmented the effect on maltose fermentation of exposure of the yeast to glucose, and enhanced certain inhibitory effects described in the sequel. Autofermentation was always found to be negligible. Samples of brewer's yeast, Rishon strain, were kindly provided by Dr Hayman of Palestine Brewery Ltd., Rishon.

Sugars. All sugars used were of good quality and supplied by B.D.H. or Schering-Kahlbaum.

Gasometers. Except where otherwise stated, fermentation was followed gasometrically using the apparatus described by Hestrin [1941]. Duplicate fermentation runs with this apparatus yielded results which were identical to within 3%. Aqueous 30% $CaCl_2$ served as the gasometer fluid.

Procedure. Mixtures were prepared for fermentation runs in 10 ml. capacity long-necked flasks and maintained in a water thermostat at 32°. 3 min. were allowed for temperature equilibration before taking zero reading. The flasks were shaken mechanically at a frequency of 70 oscillations per min. Dispersion of the yeast was further facilitated by the inclusion of several glass beads in the fermentation mixture.

Replication of experiments. All experiments reported are typical and representative of several which yielded an essentially identical result.

Units of measurement: 'gassing rate'. The measurements, being largely of relative rather than absolute importance, are presented uncorrected to standard conditions. To facilitate comparison between different experiments the gassing velocity has been expressed occasionally in terms of the maximum rate of gas evolution per unit fresh weight of yeast. Under suitable conditions this maximum is also the 'stationary' gassing rate.

Results

A. *Experiments with baker's yeast* ($V_m/V_g < 1$)

(*a*) *Fermentation rate at different initial concentrations of substrate.* The influence of substrate concentration on the fermentation process has been dealt with by a number of investigators, but their work has been largely restricted to glucose fermentation. Hopkins & Roberts [1935] found in corroboration of Slator [1908] that the gassing rate in glucose fermentation is independent of the initial substrate concentration within a wide range [cf. however, Nord & Weichherz, 1929].

Our baker's yeast, both fresh and after storage, behaved towards glucose in accordance with the findings of Hopkins & Roberts [1935]. The behaviour to maltose, on the other hand, was variable: fresh yeast cells generally fermented maltose at the same uniform

rate over a wide range of substrate concentrations. The stationary fermentation rate was always markedly below that obtained with glucose, strikingly variable with storage, and only set in after a relatively prolonged induction period. Using cell material which had been 'impoverished' by storage a definite diminution of the stationary gassing rate with increase in the initial concentration of maltose was frequently observed. Table 1 shows this retardant effect clearly. This retardation only becomes evident, it may be noted, after an initial delay.

Table 1. *Retardant effect of maltose on maltolysis by baker's yeast*

Solutions of 4·0 ml. containing 1 ml. McIlvaine's phosphate-citrate buffer *p*H 5·0, and sugar in the concentrations shown.

Initial conc. of maltose	Total yield of CO_2 in ml.* after min.							Stationary† gassing rate ml./30 min./g.
%	75	165	195	225	255	285	315	yeast
4·0	1·0	5·6	8·1	10·9 !	13·9	17·0	20·0	7·5
8·0	1·2	4·7	6·7 !	8·8	10·9	13·2	15·4	5·5
12·0	1·5	4·6 !	6·1	7·6	9·2	10·8	12·4	4·0
16·0	1·8	4·6 !	5·7	6·9	8·2	9·5	10·7	3·2

* ! indicates time of attainment of stationary gassing rate.

† Concentrations of glucose in the range of 2–16% were fermented at the uniform rate of 27·0 ml./30 min./g. yeast.

The measure of retardation of gassing rate by maltose is dependent on the physiological state of the cells. Marked retardation was frequent with cells which had been impoverished by storage. On the other hand, yeast which has been restored to full maltose fermenting capacity by treatment with glucose is not retarded or only slightly by high maltose concentration (Table 3 and Fig. 2). In view of this marked variability predictions as to the effect of concentration on maltose fermentation with types of yeast other than that used by us are hardly justified.

Mixtures of maltose and glucose were found to ferment at the same rate as glucose itself as long as an adequate concentration of glucose was maintained in the mixture. Maltose therefore did not act as an inhibitor in glucose fermentation.

The intimate mechanism of the maltose-concentration effect is obscure. This is not, however, the only known case of retardation of fermentation by a sugar. Slator [1908] found mannose a retardant of glucose fermentation, and glucose a retardant of galactose fermentation. Sobotka, Holzman & Reiner [1936] have described a retardation of hexose fermentation by pentoses. Experiments elsewhere show that under certain conditions glucose, but not maltose, may exert a retardant effect on fermentation by brewer's yeast [Hestrin, 1941].

(*b*) *Inhibition by methyl α-glucoside.* A substance analogous in certain features of structure to maltose is methyl α-glucoside. This has long been known to be an efficient retardant of invertase action [Michaelis & Pechstein, 1914] and has been recently found also to inhibit the oxidation of glucose by luminescent bacteria [Johnson & Anderson, 1938]. It seemed of interest therefore to examine its possible inhibitory action on sugar fermentations.

Under ordinary conditions the glucoside regularly failed to inhibit glucose fermentation by baker's yeast to a marked extent. Also sucrose fermentation was not noticeably affected by its presence. Raffinose fermentation, again, was slightly inhibited (experiments to be published elsewhere). Maltose fermentation, on the other hand, was strongly inhibited, both as to induction time and stationary gassing rate. Experiments showing the action of the glucoside under different conditions are summarized in Tables 2 and 3 and Fig. 1. The glucoside itself was not fermented at an appreciable rate under the conditions of these tests. Methyl β-glucoside, again, failed to inhibit maltose fermentation.

Table 2. *Substrate specificity, and influence of* p*H on inhibitory action on fermentation of methyl α-glucoside*

Suspensions of 8% yeast in mixture volumes of 4 ml. containing water or Sørensen phosphate buffer solution and 8% maltose or glucose with or without addition of glucoside. Temp. 22°. Van-Iterson-Kluyver gasometer. Intermittent shaking.

Substrate	Buffer	Methyl α-glucoside %	Total yield of CO_2 in ml. after hr. 1	3	8	24
Maltose	*p*H 4·5	0·0	0·2	1·7	9·2	23·2
	*p*H 4·5	4·0	0·1	0·7	4·8	11·1
Glucose	*p*H 4·5	0·0	5·9	16·2	—	—
	*p*H 4·5	4·0	4·1	14·4	—	—
Maltose	*p*H 7·0	0·0	0·0	0·3	2·6	11·5
	*p*H 7·0	4·0	0·0	0·0	0·2	2·4
Glucose	*p*H 7·0	0·0	5·0	14·0	—	—
	*p*H 7·0	4·0	4·8	14·3	—	—
Maltose	Water	0·0	0·1	0·5	7·2	23·0
	Water	4·0	0·0	0·0	3·9	11·4

Table 3. *Inhibition of maltose fermentation by methyl α-glucoside at different concentrations of maltose*

Mixture volume of 5·0 ml. containing 4 ml. KH_2PO_4, suitable concentrations of sugar, and 8% yeast. Yeast activated before experiment by allowing to stand in 0·9% glucose for 30 min.

Maltose %	Methyl α-glucoside %	Total yield of CO_2 in ml. after min. 30	60	90	120	150	180	Maximum gassing rate ml./30 min./g. yeast
5·0	0·0	1·8	3·8	8·2	14·2	21·6	29·4	19·5
5·0	4·0	1·0	2·7	5·4	8·7	13·0	18·2	13·0
7·5	0·0	2·1	5·6	10·5	16·7	24·0	31·8	19·5
7·5	4·0	1·2	3·7	7·5	12·3	17·6	23·6	15·0
10·0	0·0	2·3	6·2	11·5	17·6	24·9	32·5	19·0
10·0	4·0	1·9	5·0	9·0	13·6	19·2	25·3	15·2

It may be seen from the experiments reported that the action of the glucoside is a function of its concentration (Fig. 1) and also, though to a less marked degree, a function at a given concentration of glucoside of the latter's ratio to maltose (Table 3). The effect of *p*H on the inhibition is clearly shown in Table 2. In neutral phosphate solution, maltose fermentation itself is greatly depressed, but the relative effect of the glucoside is enhanced.

Since the possibility was remotely present that the inhibition was due to liberation of traces of methanol, experiments were undertaken to elucidate the effect of this alcohol on maltose fermentation. They showed that methanol in concentrations equivalent to 2–8% of glucoside does not inhibit but actually slightly favours maltose fermentation.

Attention may be drawn to the reversible character of the glucoside effect. If yeast cells which have been allowed to stand in a solution of the glucoside are separated out by centrifugation and washed they ferment pure maltose at their normal rate.

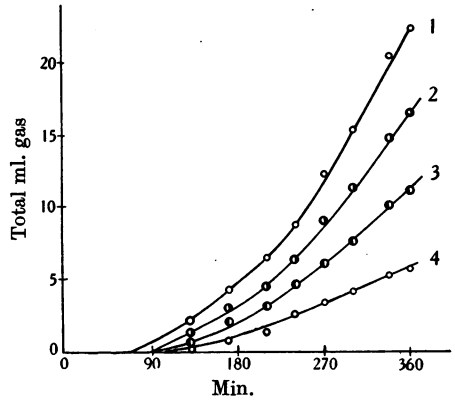


Fig. 1. Inhibition of maltose fermentation by different concentrations methyl α-glucoside. As in Table 4 but using storage-impoverished yeast. Initial concentration of maltose = 8%. Curve (1) 0·0%, (2) 2%, (3) 4%, (4) 8% glucoside.

As with the maltose inhibitory effect, that of the glucoside also is markedly dependent on the physiological state of the cells. Activation of the cells by addition of glucose

Table 4. *Effect of activation by glucose on inhibition of maltose fermentation by methyl α-glucoside*

4 ml. of mixture containing 2 ml. Sørensen phosphate *p*H 6·6, 10% yeast, and suitable concentrations of sugar. At this *p*H the glucose effect is pronounced.

Maltose %	Glucose %	Methyl α-glucoside %	Total yield of CO_2 in ml. after min. 30	60	95	110	125	170	200
8·0	0·0	0·0	0·0	1·2	3·0	4·4	6·1	12·6	17·6
8·0	0·0	4·0	0·0	0·0	0·0	0·0	0·0	0·8	1·2
8·0	0·5	0·0	2·7	4·7	8·8	10·6	12·8	20·3	25·5
8·0	0·5	4·0	3·0	4·7	6·9	8·0	9·4	13·7	17·2
0·0	0·5	0·0	2·2	2·3	—	—	2·4	—	2·4

Table 5. *Effect of previous fermentation on inhibition of maltolysis by methyl α-glucoside*

5 ml. of solutions containing 3 ml. Sørensen primary phosphate solution, 10% yeast, 8% maltose and the glucoside as indicated.

Time of glucoside addition (4%)*	Total yield of CO_2 after min. 60	90	120	150	180	200	% diminution of gas yield in 80 min. period following addition of glucoside
None	↓ 3·2	8·3	16·5	27·2	41·3	50·9	—
0 min.	↓ 0·2	1·1	4·3	10·8	18·8	26·3	88
120 min.	3·2	8·4	↓ 16·3	25·6	36·7	45·5	15

* ↓ denotes addition of glucoside.

markedly diminishes their susceptibility to inhibition by glucoside (Table 4). If the yeast cell is exposed to glucoside after maltose fermentation has set in, the inhibitory effect of the glucoside is almost negligible (Table 5).

(c) *The activation of maltolysis by zymohexoses.* The addition to maltose media of low concentrations of glucose, or of a factor such as maltase which produces glucose, has been shown to exert a remarkable stimulative influence on maltose fermentation, curtailing the induction time preceding the beginning of evolution of gas from maltose, and increasing the stationary gassing rate finally attained [Leibowitz & Hestrin, 1939; Schultz & Atkin, 1939; cf. also Blish & Sandstedt, 1937, and earlier contribution by Beijerinck, 1895].

A resolution of the dual effect of glucose addition has been achieved by variation of the initial maltose concentration. An experiment illustrating this point is summarized in Fig. 2. Glucose addition is seen to hasten the onset of maltose fermentation both at the higher and lower maltose concentration tested, but caused an appreciable increase in the ultimate gassing rate only when the higher maltose concentration inhibited fermentation.

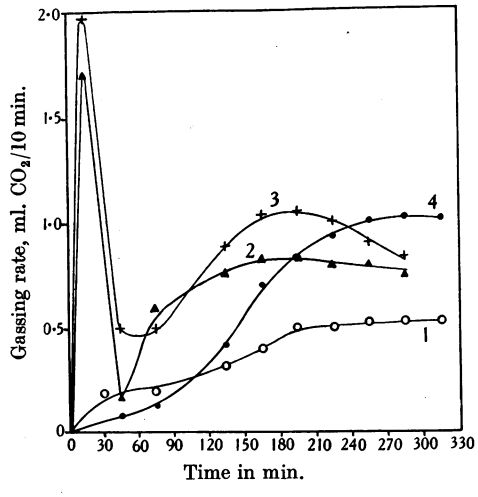


Fig. 2. Activation of maltolysis by glucose at different maltose concentrations. Composition as to buffer and yeast as in Table 1. Curve (1) 12% maltose; (2) 12% maltose and 0·5% glucose; (3) 4% maltose and 0·5% glucose; (4) 4% maltose.

The initial hump in the rate-time curves (2) and (3) of Fig. 2 is due of course to the greater fermentation rate of the rapidly disappearing glucose component. The drop in gassing rate found towards the end of the fermentation-run in the case of curve (3) is due to the approaching exhaustion of fermentable substrate.

It has been shown in the previous investigation, and it is here confirmed, that the effect of glucose is not bound up with the presence of the latter as such but persists in the yeast cell even when glucose has been removed from the medium. It seemed likely therefore that glucose acted by an influence exerted on the cell through glucolysis. Accordingly it seemed of interest to examine the effects on maltolysis of treatment of the yeast with other fermentable sugars and to compare their action with that of a normally non-fermentable sugar such as galactose. An experiment of this kind is presented in Table 6. A glance at the table shows that maltose fermentation is stimulated by fructose and mannose as well as by glucose but is not affected under the conditions of this experiment by galactose.

Table 6. *Activation of maltose fermentation by different sugars*

4·5 ml. solution containing 2·5 ml. Sørensen phosphate *p*H 6·5, 12% yeast, and suitable concentration of sugars.

Maltose %	Hexose 0·5%	Total yield of CO_2 in ml. after min. 60	120	180
6·0	None	0·0	0·1	1·0
6·0	Glucose	5·6	16·1	25·0
6·0	Fructose	4·7	11·0	20·7
6·0	Mannose	4·0	8·1	16·8
6·0	Galactose	0·0	0·0	0·8
0·0	Zymohexose*	2·0	2·0	2·0
0·0	Galactose	0·0	0·0	0·0

* Zymohexose = glucose, fructose or mannose.

(*d*) *p*H *relations*. The favourable effect of moderately acid *p*H on maltose fermentation was discovered by Willstätter & Bamann [1926] and has since been confirmed by different investigators [Sobotka & Holzman, 1934; Genevois, 1937; Schultz & Atkin, 1939]. The baker's yeast used in the present investigation showed the favourable effect of acid *p*H very clearly (e.g. in Table 3).

Experiments carried out towards the conclusion of this investigation revealed that the true nature of the *p*H-activity curve is obscured in the neutral range when a comparatively weak buffer is used. Thus, in neutral Sørensen phosphate, maltose fermentation is delayed but still possible. It could be shown, however, that the *p*H of these mixtures suffers a shift to the acid side as fermentation of maltose sets in. Inclusion of a little glucose in the fermentation mixtures hastens the onset of the acid shift [cf. Mirski & Wertheimer, 1939].

Using McIlvaine citrate-phosphate buffer, the acid shift from neutral was found to be largely eliminated. In this buffer precise determination of the variation of stationary gassing rate with *p*H in a sufficiently wide range of *p*H values becomes possible (*p*H 3·0–7·0). Typical experiments showing the dependence of fermentation on *p*H in adequately buffered medium are summarized in Fig. 3 (curves I and II).

It is apparent that whereas glucolysis by living baker's yeast is independent of the *p*H within the tested range of 3·0–7·0, maltolysis is optimal at *p*H 5·0, still considerable at *p*H 3·0, but completely suppressed under the conditions of the test at *p*H 7·0. Methyl α-glucoside was not fermented by fresh baker's yeast at any *p*H whatsoever under the conditions of the experiment summarized in Fig. 3.

Promaltose. It is important to note, particularly, in view of certain analytical methods of differentiating between maltose and glucose, that the negative fermentation reaction of baker's yeast to maltose and methyl α-glucoside at neutral *p*H is extremely

labile. Fresh, stored, and glucose-activated cells of baker's yeast failed to ferment maltose and the glucoside at neutral *p*H, but slight ability to ferment maltose at neutral *p*H was acquired by the yeast on prolonged incubation with maltose (over 8 hr.). Ability to ferment both maltose and glucoside at neutral *p*H was also conferred on the fresh yeast by drying (Table 7).

Table 7. *Fermentation of maltose and methyl α-glucoside by fresh and dried baker's yeast at neutral reaction*

Solution, 4·0 ml. containing 2·0 ml. McIlvaine phosphate-citrate buffer *p*H 7·0, 5% sugar, and 12% of fresh or air-dried baker's yeast.

Yeast	Substrate	Total yield of CO_2 in ml. after min. 20	40	60	95	115
Fresh	Maltose	0·0	—	0·0	—	0·0
Fresh	Methyl α-glucoside	0·0	—	0·0	—	0·0
Dried	Maltose	6·1	14·4	21·6	—	39·5
Dried	Methyl α-glucoside	1·2	2·4	3·6	6·4	8·2

Moreover, when fresh baker's yeast was analysed for maltase content by autolysis with ethyl acetate, as described by Willstätter & Steibelt [1921], a slight amount of

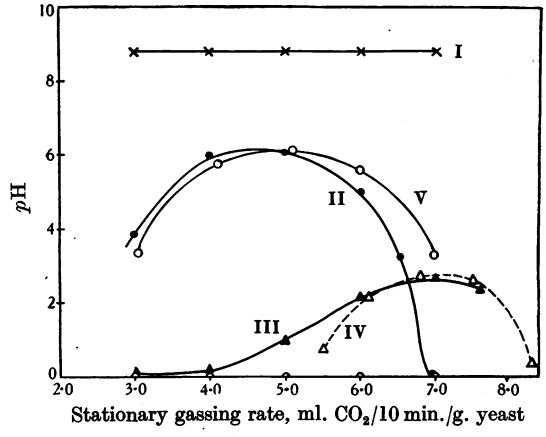


Fig. 3. Fermentation of sugars at different *p*H values. Solution, 5 ml. containing 2 ml. of McIlvaine citrate-phosphate buffer, 6% yeast, and 6% of sugar substrate. Temperature 32°. Curve I, glucose fermentation by baker's yeast. Curve II, maltose fermentation by baker's yeast. Curve III, methyl α-glucoside fermentation by brewer's yeast. Curve IV, relative activity-*p*H curve of yeast maltase [from data of Willstätter & Bamann, 1926]. Curve V, maltose fermentation by brewer's yeast. Values plotted are relative. For absolute figures of gassing rate multiply by 31.

maltase was found to be liberated even though the fresh sample had given a negative reaction for maltose fermentation at neutral *p*H. The order of activity of this maltase solution to maltose, and the glucoside may be seen in Table 8.

Table 8. *Maltase of baker's yeast autolysate*

Autolysate prepared after Willstätter & Steibelt [1921] from baker's yeast negative to maltose at *p*H 7·0 in fermentation test. Solutions of 5·0 ml. total vol., containing 1·0 ml. autolysate, 2·0 ml. Sørensen phosphate buffer *p*H 7·0, and sugar as indicated. Incubation at 37°. Reducing power determined according to Bertrand on 2·0 ml. samples.

Substrate	Conc. in %	% hydrolysis after 24 hr.
Maltose	4	32
	8	16
Methyl α-glucoside	4	21

A hypothesis which would explain these apparently paradoxical findings is that fresh baker's yeast contains maltase in the form of a zymogen which is converted into active maltase (=α-glucosidase) by treatments such as drying or autolysis. It is not unlikely that a similar explanation will apply to analogous findings with other oligase enzymes, e.g. the presence of lactase in preparations of *E. coli mutabile* variants which were inert to lactose [Deere, Dulaney & Michelson, 1939], trehalase in yeast which did not ferment trehalose [Myrbäck & Örtenblad, 1936], sucrase in autolysates of *Schizosaccharomyces octosporus* which does not ferment sucrose [Hofmann, 1934], cellobiase in preparations of lactose yeast which had failed to ferment cellobiose [Neuberg & Hofman, 1932], maltase in yeast which failed under anaerobic conditions to ferment maltose [Kluyver & Custers, 1940].

B. *Experiments with brewer's yeast* ($V_m/V_g \gtrless 1$)

(*a*) $V_m/V_g = 1$. Brewer's yeast, in media of the type employed in the foregoing, generally ferments maltose and glucose at the same uniform rate and after roughly equal, very brief inductions. With 9 out of 10 lots of yeast tested under these conditions the V_m/V_g ratio approximated closely to unity, and was practically independent of the concentrations of substrate employed for the comparison (2–12 %).

As was to be expected from considerations set forth in the introduction, maltose fermentation by this type of yeast was insusceptible of inhibition or activation by specific activators and inhibitors of maltolysis. Methyl α-glucoside or maltose in high concentration did not inhibit, and hexose addition did not activate, maltose fermentation by Richon brewer's yeast.

(*b*) $V_m/V_g > 1$. An exceptional lot of Richon yeast actually fermented maltose more rapidly than glucose. Experiments showing the fermentation of maltose and glucose by this yeast lot at different initial substrate concentrations and before and after ageing of the yeast are summarized in Fig. 4. It will be noted that ageing enhanced the relative superiority of the fermentation rate of maltose. It is also apparent that the ratio V_m/V_g is dependent on the initial substrate concentration at which the fermentation rates are determined. In contrast to ordinary brewer's yeast lots which fermented glucose as well as maltose at a uniform rate in a range of 2–12 %, the unique sample in question fermented glucose less readily at 8 % than at 4 % concentration, whereas maltose in the same range was fermented by the sample at a uniform rate.

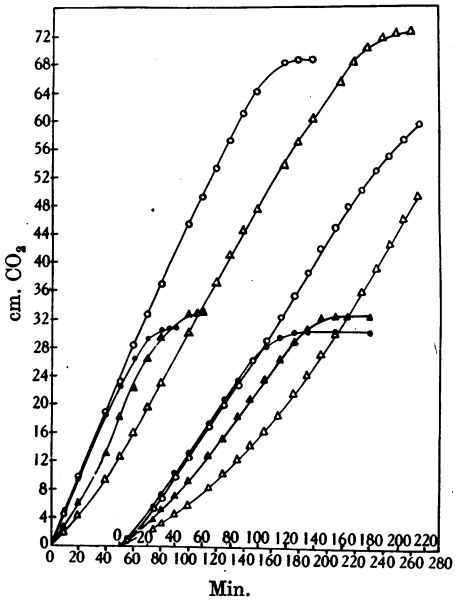


Fig. 4. Fermentation of maltose and glucose by exceptional lot of brewer's yeast. Solutions, 4·0 ml. containing citrate-phosphate buffer *p*H 6·8 prepared according to Willstätter & Bamann [1926] and at 20-fold final dilution, 10 % Richon brewer's yeast, and designated concentrations of sugar. Left hand curves, fermentation by cells fresh from brewery; right hand curves, fermentation by same cells after storage on ice for 2 weeks. (1, •—•) maltose 4 %, (2, ▲—▲) glucose 4 %, (3, o—o) maltose 8 %, (4, △—△) glucose 8 %.

V_m/V_g fermentation ratios greater than unity have been reported previously by Trautwein & Wassermann [1929], Yamasaki [1930] and by Sobotka & Holzman [1934]. Working with variously aged samples of Richon yeast and in media of injuriously high buffer salt concentration, e.g. in undiluted citrate-phosphate prepared according to Willstätter & Bamann [1926], the present authors have observed V_m/V_g values consider-

ably greater than unity with fair regularity [Leibowitz & Hestrin, 1940; Hestrin, 1941]. The effect on fermentation under these conditions of ageing and of substrate concentration was qualitatively as noted in Fig. 4 for the 'exceptional yeast lot' in relatively low salt medium, and need not be reported separately.

The significance of these findings for direct fermentation theory remains to be considered. The interpretation of the evidence to hand is, however, problematic, and complicated by the following considerations:

(1) V_m/V_g ratios greater than unity have been observed in buffer of *p*H 6·8. At this *p*H, however, the activity of maltozymase, at least in baker's yeast, appears to be completely suppressed.

(2) In all the experiments where maltose fermented more rapidly than glucose, increased concentration of glucose led to a lowered rate of glucolysis. It is therefore conceivable that V_m/V_g values greater than unity were only obtained here because in the presence of excess of glucose the full glucolytic ability of the yeast failed to come into play.

Analogous possibilities must also be considered in connexion with other findings of superior disaccharide fermentability, reported in the literature (Willstätter & Oppenheimer [1921] and Hestrin [1941] for lactose; Wright [1936] for sucrose; Myrbäck & Örtenblad [1937] and O'Connor [1940] for trehalose).

(*c*) p*H relations*. Experiments comparing the dependence of maltose fermentation on *p*H by brewer's and baker's yeast with that of methyl α-glucoside fermentation by brewer's yeast and all three with the known *p*H function of dissolved maltase are summarized in Fig. 3. It is evident from the figure that the *p*H-activity curves of cell-free and cell-bound maltase (=α-glucosidase) are essentially similar (curves III and IV respectively). The relatively slower drop in activity of cell-bound maltase at values below *p*H 6·0 is probably only apparent and due to a modification of intracellular *p*H in this range by cell-formed buffer substances.

The presence of active maltase in fresh cells of Richon yeast is confirmed by the positive fermentation test of this yeast to methyl α-glucoside. This conclusion further suggests a ready explanation of the difference in the *p*H-activity curves of maltose fermentation by baker's and brewer's yeasts respectively (curves II and V). It is to be expected that in the presence of α-glucosidase maltose will be fermented at *p*H 7·0 which is in the optimum range for this enzyme. The experiment summarized in curve V shows that this is indeed the case. The true *p*H dependence of *direct* maltose fermentation is presumably masked in the case of brewer's yeast by the activity in the neutral range of cell maltase.

Discussion

Experiments reported in the present and in previous investigations [Leibowitz & Hestrin, 1939; Schultz & Atkin, 1939; 1940; Kluyver & Custers, 1940] confirm that under suitable conditions maltose fermentation by yeast may be selectively inhibited or accelerated by agents which are without known similar or comparable influence on either glucozymase or maltase. The differential effects of certain sugars, hydrogen ion concentration, and oxygen on the fermentation of maltose and glucose by baker's yeast are compared in the following table.

	Effect on fermentation of	
Agent	Glucose	Maltose
Maltose	None	Inhibition (variable)*
Methyl α-glucoside	None	Inhibition
Zymohexose (traces)	None	Activation
O_2	Inhibition	Activation†
*p*H 4·5	Optimum	Optimum
*p*H 7·0	Optimum	Complete inhibition

* In concentration range providing adequate amount of substrate.
† Schultz, Atkins & Frey [1940].

Since the listed reagents affect maltose fermentation in an essentially different manner from glucose fermentation it is obviously necessary to assume that maltose fermentation involves a specific rate-limiting component which is absent from glucose fermentation. The question arises whether the specific component is maltase. Discussion of this question must be preceded, however, by consideration of the possibility that the different effects noted are manifestations of specific effects on the permeability apparatus involved in maltose absorption, rather than effects on the fermentation enzymes themselves.

The suggestion has been entertained that O_2 and glucose influence the rate of gas evolution from maltose by affecting the rate at which maltose diffuses into the cell [Schultz *et al.* 1939; 1940]. Improved permeability could only affect the gassing rate, however, where the rate of diffusion is the limiting factor. Available evidence weighs against this conclusion. The measurements of Sobotka *et al.* [1936] show that maltose diffuses into baker's yeast at least one-half as rapidly as glucose. But maltose is frequently fermented by glucose-activable yeast with a velocity considerably below half that of glucose. The limiting factor cannot therefore be the rate of diffusion. This conclusion is further strengthened by the observation that maltose fermentation has a high temperature coefficient [Slator, 1908] and also by kinetic data advanced above. It is to be expected on the view that permeation is the rate-limiting process that increase in the substrate concentration would lead to increased permeation, a resultant increase in gassing rate and a corresponding lessening of the yeast's susceptibility to activation by glucose. None of these predictions is realized in practice (cf. Tables 1 and 3, and Fig. 2). It seems correct to conclude therefore that the zymohexoses and O_2 do not affect maltose fermentation through their action on permeability.

The above conclusion may be further extended to the substrate-specific retardant effect of methyl α-glucoside on maltose fermentation. If permeability were rate-limiting for maltose fermentation in the presence of a given inhibitor, activators of reaction steps subsequent to the diffusion process should fail to affect the gassing rate. It has been shown, however, that glucose can activate maltose fermentation both in the presence and absence of methyl α-glucoside (Table 4). Since it has been concluded that glucose acts on the fermentation sequence at a stage subsequent to the diffusion process it becomes necessary to assume also that methyl α-glucoside acts on maltose fermentation at a stage identical with or subsequent to that acted on by glucose, i.e. a stage other than that of the permeation process.

The view that hydrolysis by maltase is the rate-limiting component in the fermentation of maltose by baker's yeast necessitates the assumption that zymohexose, O_2, methyl α-glucoside, and maltose in high concentration affect the rate at which maltose is fermented through specific actions on cell maltase. None of these substances affects the activity of maltase in solution in a manner comparable to their action on maltose fermentation. Glucose which stimulates maltose fermentation is itself formed from maltose by maltase but its action on maltase is that of an inhibitor [Kuhn, 1923]. Stimulation of maltase activity by O_2 has never been demonstrated. Maltose and methyl α-glucoside, again, are substrates of yeast maltase. It has been shown that increase of the maltose concentration within a wide range does not inhibit but leads to an increase in the rate at which glucose is liberated by maltase [Willstätter, Kuhn & Sobotka, 1924]. The view that methyl α-glucoside affects maltose fermentation by competing for maltase enzyme with maltose is superficially attractive but fails to explain an important characteristic of the inhibition: the virtual absence of inhibitory response when methyl α-glucoside is added after maltose fermentation has already fully set in (Table 5). It is to be expected, moreover, in this view that methyl α-glucoside would itself be split by maltase and hence be slowly fermented. The fresh baker's yeast failed, however, under the conditions of the experiments to ferment methyl α-glucoside. The absence of such fermentation must be regarded as evidence that the baker's yeast cells contained no active maltase (=α-glucosidase).

Further support for the view that hydrolysis by maltase is not the limiting factor of, or indeed necessary to, maltose breakdown is provided by consideration of the *p*H-activity curves of maltose, glucose, and methyl α-glucoside fermentation respectively. Fig. 3 shows that our baker's yeast though able to ferment maltose in the tested *p*H range 3·0–6·0 is normally unable in adequately buffered medium to ferment this disaccharide at *p*H 7·0, the known optimum *p*H of both dissolved and cell-bound maltase. Glucose is fermented at *p*H 7·0 with optimum velocity.

The specific activating effect of zymohexoses and the retarding action of saccharides such as methyl α-glucoside on maltolysis becomes comprehensible, on the other hand, if the view is accepted that maltose fermentation involves the specific participation of activators which are formed in the cell during and by the breakdown of fermentable sugar [Leibowitz & Hestrin, 1939]. The activation, it may be noted, persists long after the last trace of glucose has been exhausted by fermentation. Its dependence on a substance formed during the fermentative breakdown of hexose rather than on hexose as such is confirmed by the finding that only fermentable hexoses but not galactose activate maltose fermentation (Table 6).

The effect of glucose is complex: in weakly buffered media near neutral *p*H, glucose fermentation causes a shift in *p*H to greater acidity [Mirski & Wertheimer, 1939] and this favours maltose fermentation. There remains, however, at acid *p*H a residual activation effect due to glucose [Schultz & Atkin, 1939]. This is manifested at all maltose concentrations through a curtailment of the induction period which precedes the onset of gas evolution, but at high maltose concentration may also be manifested through an increase of the stationary gassing rate ultimately attained (Fig. 2).

Little is known concerning the nature of the specific catalysts of maltolysis. The discovery that O_2 plays an activating role in maltose fermentation [Schultz *et al.* 1940; Kluyver & Custers, 1940] may throw light on this subject. Schultz *et al.* [1940] mention that in the presence of traces of glucose the effect of O_2 is masked. It is therefore possible that glucose acts functionally as a substitute for O_2. It has been shown that both O_2 and glucose exert a depressant effect on the concentration of phosphate in the yeast cell [Macfarlane, 1936; Johnson, 1941]. The possibility that their common stimulative action on maltolysis is mediated through an effect on phosphate will therefore deserve investigation.

A further weapon for the elucidation of the catalyst complex involved in maltolysis is provided by methyl α-glucoside. This reagent appears to constitute a specific retardant of maltose fermentation by baker's yeast. Its effect is not given, moreover, by methyl β-glucoside or by methanol. It has been shown that when methyl α-glucoside is added to the maltose-yeast mixture after the beginning of gas evolution its inhibiting efficiency is largely abolished (Table 5). Cells fresh from a previous maltolysis are relatively indifferent to inhibition by methyl α-glucoside presumably because such cells already possess a ready store of the catalyst system whose formation would have been retarded in the presence of methyl α-glucoside. It seems correct to conclude therefore that methyl α-glucoside acts not as a retardant of the main cycle of maltolysis but rather as a specific retardant of a catalyst-forming side-reaction. A possible role for O_2 in this side-reaction is suggested by the observation that methyl α-glucoside acts also as a retardant on oxidation systems [Johnson & Anderson, 1938].

Methyl α-glucoside, which is also a sucrase inhibitor, slightly retarded raffinose fermentation but was without noticeable effect on sucrose fermentation by a baker's yeast. It therefore seems correct to conclude that invertase action was rate-limiting in raffinose fermentation but not in sucrose fermentation by this baker's yeast.

Work on the nature of maltolysis must further centre in future on the identification of the metabolite in the formation of which the different reaction paths of maltolysis and glucolysis are assumed to merge. It has been suggested previously, in view of the findings

of Willstätter & Rohdewald [1937; 1940], that this metabolite is glycogen [Leibowitz & Hestrin, 1939]. The finding that certain preparations of phosphorylase may act on maltose as well as on higher polysaccharides though not on glucose [Hanes, 1940; cf. however, Cori, Colowick & Cori, 1938] raises the further possibility that the unnamed first common metabolite of glucolysis and maltolysis is glucose-1-phosphate. The first step in maltolysis would on this view be a phosphorylysis of the maltose molecule.

The relative importance of the maltase-glucozymase complex and of maltozymase, respectively, in maltose fermentation by yeast is obviously dependent on a variety of factors including *p*H and temperature [cf. Leibowitz & Hestrin, 1939]. The two systems are not, however, necessarily of the nature of competitors, but act each in separate though overlapping *p*H ranges. Maltozymase, as is shown by Fig. 3, is exclusively responsible for maltose fermentation at *p*H values below 4·0; a maltase-glucozymase system is responsible for any maltose fermentation at *p*H values above 6·8.

The failure of a yeast sample to ferment maltose at *p*H 7·0 is valid evidence that under the conditions of the test maltase is not actively present in the cells, but does not constitute proof that preparations of the cells will be inactive to maltose. Experiments summarized in Table 7, show that yeast cells which failed to ferment maltose at *p*H 7·0, and methyl α-glucoside at any *p*H, acquire ability to ferment these substrates at neutral *p*H after being dried. This finding suggests that maltase may exist in the cell in an inactive form. This maltasogen is presumably convertible into active maltase through dissolution of the cell structure by drying or autolysis (cf Table 8). The much discussed unreliability of in vitro analysis as a method of determining maltase activity in vivo is thus further confirmed but in an unexpected direction. It has been widely accepted, hitherto, that absence of a given enzyme in cell preparations does not constitute proof of its absence in life. The possibility has also to be considered, however, that an enzyme may become active in vitro which was inactive in the living cell.

Summary

Experiments are reported dealing with the relative role and nature of the direct and indirect enzyme mechanisms of maltose fermentation in yeast.

A. *Baker's yeast*

(1) Different agents may selectively inhibit or stimulate maltose fermentation by baker's yeast without similar effect on glucolysis or maltase activity.

(*a*) Maltose fermentation was stimulated by any of the zymohexoses but not by galactose.

(*b*) Methyl α-glucoside is a powerful substrate-specific retardant of maltose fermentation by baker's yeast. The inhibition by methyl α-glucoside is completely reversible. Its intensity increases with increasing concentration of glucoside. Methyl β-glucoside and methanol in equivalent concentration did not inhibit maltose fermentation.

(*c*) High concentrations of maltose may inhibit the rate at which maltose is fermented by aged baker's yeast but do not similarly affect glucose fermentation or maltase activity in vitro. Addition of glucose traces abolished the retardant effect of high maltose concentration.

(*d*) The *p*H-activity curve of cell-bound maltase was largely identical with that of cell-free maltase, distinct from that of glucozymase, and actually opposite in trend to the *p*H-activity curve of maltose fermentation.

(*e*) Maltose is vigorously fermented by living baker's yeast at acid *p*H values which preclude hydrolysis by either cell-free or cell-bound maltase, but was not fermented at all by the same cells at neutral *p*H, which is optimal for maltase.

The conclusion follows that maltose fermentation by baker's yeast is direct.

(2) The view is advanced that the maltozymase complex of yeast involves specific maltose fermentation catalysts and that these are generally formed in the cell during maltose fermentation.

(*a*) Methyl α-glucoside only retarded maltolysis when added to the fermentation mixture before maltose fermentation had become vigorous.

(*b*) Traces of glucose markedly curtailed, but never entirely abolished, the induction period preceding the onset of the stationary phase of maltose fermentation.

(3) Baker's yeast cells failed to ferment maltose at neutral *p*H, and methyl α-glucoside at any *p*H, but acquired ability to ferment these substrates at *p*H 7·0 when dried. The fresh cells yielded active typical maltase on autolysis. It seems necessary to conclude that these yeast cells may contain maltase in inactive forms (maltase zymogens).

B. *Brewer's yeast*

(4) Maltose fermentation by brewer's yeast differed in several respects from that by baker's yeast.

(*a*) Ordinarily samples of brewer's yeast fermented maltose and glucose at an equal rate. As expected, maltose fermentation was here found insusceptible of inhibition by methyl α-glucoside or of activation by traces of hexose.

(*b*) At high substrate concentration, maltose was in certain cases fermented more rapidly than glucose.

(*c*) Maltose fermentation by brewer's yeast occurs at neutral as well as at acid *p*H (3·0–7·0). The same cells ferment methyl α-glucoside, vigorously in the neutral range, but not at all below *p*H 4·0. They may be concluded to contain active maltase.

It is suggested that at acid reaction, brewer's yeast ferments maltose by means of a direct fermentation mechanism (maltozymase) and that an indirect fermentation mechanism—maltase + glucozymase—is solely or largely responsible for fermentation of maltose in the neutral range.

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