XXXI. SELECTIVE FERMENTATION. ALCOHOLIC FERMENTATION OF MIXTURES OF GLUCOSE AND FRUCTOSE BY BREWER'S AND SAUTERNE YEASTS.

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IN recent years there has been a revival of interest in the phenomena of selective fermentation of mixtures of glucose and fructose by brewer's and other yeasts. Contributions have appeared from Willstätter and Sobotka [1922], Fernbach, Schoen and Mori [1927], Ivecovich [1930], Hopkins [1928], and Sobotka and Reiner [1930, 2]. Convenient summaries of the known phenomena appear in each of the last two papers. Broadly speaking, the facts calling for explanation are as follows:

(1) that glucose and fructose in separate solutions are fermented at approximately equal rates by living yeasts;

(2) that in mixtures of the two sugars, glucose is fermented faster than fructose by brewer's and most yeasts, but the reverse is the case with certain Sauterne yeasts; the selectivity was expressed by Hopkins [1928] by the formula

$$K_{G/F} = \frac{\ln a_g - \ln (a_g - x_g)}{\ln a_f - \ln (a_f - x_f)}$$

where a_g and a_f are the initial concentrations of glucose and fructose respectively, and x_g and x_f the concentrations fermented in a given time;

(3) that fructose alone is specifically esterified at least as rapidly as glucose by yeast juice or zymin prepared from brewer's yeast, and more rapidly in the presence of suitable concentrations of inorganic phosphates.

From a consideration of facts (1) and (2), it must be concluded that the total rate of fermentation is controlled by one of the reactions succeeding the stage at which selection occurs. The later reactions are apparently the same for both hexoses. The fact of selective fermentation, indeed, strongly supports the idea that the first reaction of fermentation in the living yeast cell is more rapid than a subsequent one. This is in agreement with the results of Harden and Young's work with yeast juice. Since such a majority of yeasts prefer glucose, Sauterne yeasts should be regarded as the exception. Neglecting this for the present, and considering together facts (2) and (3), the anomaly seems to be presented by brewer's and most other yeasts, and the author's attention was firstly directed to this.

It was thought possible that the rate of diffusion of one sugar into the

cell may be influenced by reaction products of the other sugar or even that one sugar may be produced from the other within the cell. For example, Harden and Young [1910] found that fructose is produced from hexose-diphosphate by the action of an enzyme, hexosephosphatase, in yeast juice. If such fructose is produced within the living cell, it would be produced at a definite rate per cell. More than one explanation of the selective action of brewer's yeast could be advanced on this hypothesis. The production of one sugar from the other *via* hexosephosphate at a fixed rate per cell should be independent of the concentrations of glucose and fructose in the external medium over wide range, so that the value of K should vary if widely differing initial proportions of glucose and fructose are used. This, however, was found not to be the case (Exp. 1).

It was shown by Kuhn and Münch [1925] that saccharase is of two types, that of brewer's yeast for example which is inhibited by fructose, and that of *e.g.* *Aspergillus oryzae* which is inhibited by glucose. Weidenhagen [1930] terms these two types respectively β -fructosidase and α -glucosidase. The possibility arises that the saccharases of brewer's and Sauterne yeasts are respectively of these two types, and in some way function to inhibit the fermentation of the respective sugars with which they may be assumed to combine. Investigation gave the following results.

(1) The saccharases of both brewer's and Sauterne yeasts were inhibited about twice as much by fructose as by glucose (Exp. 2). This was referred to by the author in a previous paper [Hopkins, 1930].

(2) Brewer's yeast, treated to reduce its saccharase activity relatively to its fermentative power by the method of Willstätter, Lowry and Schneider [1925], exhibited much the same selective power as before (Exp. 3). Sobotka and Reiner [1930, 2] have recently reported a similar result.

(3) Zymin prepared from brewer's yeast ferments glucose faster than fructose in a mixture, and this preference was undiminished by the addition of saccharase in the form either of a crude yeast autolysate (too old to contain any co-zymase) or of purified saccharase (Exp. 4). It should be noted, however, that the selective preference for glucose exhibited by zymin may be due simply to the production of fructose by hexosephosphatase.

The rate of fermentation of glucose and fructose in separate solutions. The approximate equality of the rates of fermentation in separate solutions by living yeast has been proved by experiments in which the reactions were of the zero-molecular type, *viz.* the rates measured were functions of the concentration of yeast and of one of the later reactions of the fermentation. Unless the sugar concentration is low enough to introduce a new limiting factor, no difference between the rates for the two separate sugars is likely to be found. It was found, however, that:

(1) Brewer's yeast ferments glucose faster than fructose when the concentration of sugar in the fermenting mixture is well below 1 %, whereas approximate equality in rate was observed above 1 % concentration.

(2) Sauterne yeast ferments glucose more slowly than fructose at any concentration up to 10 % (the maximum tested), but more decidedly so as the concentration falls (Exp. 5).

Influence of temperature on the value of $K_{G/F}$. It was observed by Hopkins [1928] that the selectivity of brewer's yeast was greater as temperature was lower. This has been confirmed, and values of $K_{G/F}$, 2.59 at 8°; 2.48 at 20°; 2.30 at 31° were obtained. Sauterne yeast yielded corresponding values for $K_{G/F}$, 0.051, 0.071, 0.098 at these temperatures respectively. The values of K were compared as far as possible at corresponding stages of the fermentations (Exp. 6). These results led to the hypothesis that brewer's yeast exerts a selective preference for that constituent of fructose which increases in proportion to rise in temperature. It is well known that the specific rotation of fructose becomes less negative by 0.64° per degree rise in temperature. This is explained by a change in the relative proportions of the constituent forms of fructose.

The mutarotation of fructose and glucose when partly fermented by the yeasts. Fructose, in accordance with the above hypothesis, mutarotated in the positive direction when brewer's yeast was used, and in the negative direction when Sauterne yeast was used. Glucose when partially fermented by brewer's yeast, showed a mutarotation in the positive direction, the value of $[\alpha]_D^{20}$ being + 49°. Willstätter and Sobotka [1922] obtained a similar result with a Munich brewery yeast. Again, Sauterne yeast acted in the converse manner, the glucose possessing $[\alpha]_D^{20} + 54^\circ$ at interruption.

In any explanation of the general phenomena of selective fermentation these results must be taken into account. The simplest inference to be drawn from the mutarotations of glucose is that brewer's yeast selectively prefers the α -form ($[\alpha]_D + 110^\circ$) and Sauterne yeast the β -form ($[\alpha]_D + 19^\circ$) of glucose. On this assumption the values of $K_{\alpha/\beta}$ for the brewer's yeast are 1.4 to 1.5 at the stage of 30 to 40 % of total glucose fermented. Willstätter and Sobotka's result for the corresponding stage of fermentation was only slightly higher. The selective action of the yeasts on fructose constituents is more significant. It may be that only α - and β -forms of normal fructose exist in an aqueous solution. β -Fructose has a specific rotation of -130° [Nelson and Beegle, 1919], the other form has not been isolated, but is presumably present only in small proportion at low temperatures, but increasingly with rise of temperature. However, the striking change in optical rotation of fructose with change of temperature, a phenomenon not paralleled in glucose or the other familiar sugars, strongly suggests that the unidentified form is one the formation of which from normal fructose involves a not inconsiderable energy change. Brown and Pickering [1897] found that 1 g. of glucose (α) evolves from 0.7 to 0.9 calorie in mutarotating to equilibrium, whereas 1 g. of fructose absorbs from 4.6 to 5.4 calories (probably only about 0.1 g. of fructose actually changes to the γ -form). Normal fructose, according to the now generally accepted formula of Haworth consists of an amylene oxide or δ -ring (pyranose).

The other form may be a butylene oxide or γ -ring (furanose). The latter probably possesses a + rotation as would be expected from considerations of the specific rotations of the tetramethyl δ - and γ -fructoses. The application of Hudson's lactone rule to γ -fructose would also indicate a + rotation. If this is so this form of fructose, if present, would be present in very low proportion in aqueous solutions of fructose at ordinary temperatures. (It should be noted that Morgan and Robison [1928] found that the hexose of hexosediphosphate is γ -fructose.)

The hypothesis is now advanced that brewer's and presumably most yeasts are specific for the normal aldo-hexoses, glucose and mannose, and for this unidentified, but probably γ -form of fructose. If, as seems probable, this latter is present only in relatively small proportion in fructose solutions, it would necessarily be taken up slowly in a selective fermentation of a mixture of glucose and fructose. Conversely, Sauterne yeasts are specific for normal fructose (or β -fructose on the simple theory of only α - and β -forms) and ferment glucose specifically more slowly than normal fructose. If this hypothesis should prove correct, the problem of the selective fermentation of these sugars by various yeasts resolves itself into a more normal one, *viz.* that brewer's and most yeasts are specific for certain hexoses, those derived directly from maltose, sucrose, *etc.*, and Sauterne yeasts for normal fructose as it occurs in grapes.

EXPERIMENTAL.

The glucose and fructose used in these experiments were found to give $[\alpha]_D^{20} + 52.6^\circ$ and -93.0° respectively. The brewer's yeasts were all top fermentation yeasts obtained from Scottish breweries. Exps. 5 to 8 (Tables IV, V, VI and I, Nos. 11-20) were performed within a few weeks of each other with yeast obtained from the same source. The Sauterne yeast used in the same series was isolated from a bottle of Sauterne wine (1928), from which the yeast used in Exp. 2 had been previously isolated in 1928. It was grown in a medium of grape must and fructose.

Exp. 1. Selective fermentations by brewer's yeast with varying initial proportions of glucose and fructose.

Fermentations with yeasts A and B were performed at 25° , and samples withdrawn at intervals, centrifuged and filtered. The polarimetric readings were taken at 20° after allowing time for the rotations to stabilise at that temperature, and the reducing powers by the method of Lane and Eynon. The results are given in Table I (1-7).

Exp. 2. Inhibition of the saccharase of brewer's and Sauterne yeasts by glucose and fructose respectively.

The inversions were performed at 20° and all polarimetric readings were made at the same temperature which was never allowed to change.

Table I. *Selective fermentation of glucose and fructose by various types of yeast and under various conditions.*

Exp.		Yeast and temp.	Time (hours)	% fer- mented of total sugars	Sugars unfermented		$K_{G/F}$
					Glucose	Fructose	
Exp. 1.	1	Brewer's A 25°	—	0 39 63	10.06 5.79 3.25	2.45 1.88 1.41	— 2.08 2.03
	2	„	—	0 54 71	6.30 1.99 0.96	5.98 3.56 2.61	— 2.22 2.26
	3	„	—	0 77	2.64 0.24	10.00 2.66	— 1.79
	4	Brewer's B 25°	—	0 80	9.98 1.725	1.11 0.465	— 2.02
	5	„	—	0 77	9.00 1.624	1.84 0.816	— 2.10
	6	„	—	0 23 77	5.04 3.56 0.833	5.16 4.36 2.22	— 2.07 2.13
	7	„	—	0 31	1.50 0.721	8.40 6.15	— 2.30
Exp. 3.	8	Brewer's C Normal	0 2½ 5½	0 47 79	4.93 1.85 0.40	4.88 3.32 1.64	— 2.54 2.32
	9	Same (acid treatment)	0 3 5½	0 26 45	4.95 3.24 2.06	4.90 4.10 3.34	— 2.38 2.29
	10	Same (alkali treatment)	0 2½ 5½	0 33 56	4.93 2.70 1.42	4.88 3.84 2.88	— 2.51 2.36
Exp. 6.	11	Brewer's D 9°	0 7 22	0 25 70	4.23 2.61 0.61	4.17 3.67 1.91	— 3.80 2.48
	12	20°	0 4	0 37	4.79 2.40	4.72 3.57	— 2.48
	13	31°	0 4 6	0 48 70	4.94 1.90 0.77	4.86 3.23 2.17	— 2.33 2.30
	14	8°	0 23 52	0 31 71	4.13 2.34 0.536	4.07 3.29 1.85	— 2.67 2.59
	15	34°	0 4 7½	0 38 71	4.13 2.04 0.64	4.07 3.02 1.76	— 2.36 2.22
	16	Sauterne (isolated 26. x. 29) 8°	0 32	0 43	4.09 3.90	3.98 0.72	— 0.028
	17	34°	0 5	0 50	4.09 3.73	3.98 0.27	— 0.034
	18	Sauterne 7°	0 8 19	0 13 25	9.26 8.82 8.26	2.06 1.07 0.22	— 0.074 0.051
	19	20°	0 8	0 22	9.99 8.99	2.21 0.50	— 0.071
	20	31°	0 8	0 12	10.35 9.79	2.28 1.29	— 0.098

1. 100 cc. of 0.146 *M* sucrose + 2.0 g. of pressed yeast + 4 cc. chloroform.
2. 100 cc. of 0.146 *M* sucrose } + 4.0 g. of pressed yeast + 8 cc. chloroform.
+ 100 cc. of 0.146 *M* glucose }
3. 100 cc. of 0.146 *M* sucrose } + 4.0 g. of pressed yeast + 8 cc. chloroform.
+ 100 cc. of 0.146 *M* fructose }

25 cc. samples were withdrawn and the reaction stopped by addition to 2 cc. of *N* NaOH and 1 cc. of alumina cream.

Table II. *Inhibition of inversion of sucrose by yeasts in the presence of glucose and fructose.*

		Polarimetric readings (2 dm. tube) at 20°					
		Sucrose + glucose			Sucrose + fructose		
		(0.146 <i>M</i>)	(0.146 <i>M</i>)		(0.146 <i>M</i>)	(0.146 <i>M</i>)	
		Sucrose (0.146 <i>M</i>)	Inhibition %		Inhibition %		
Brewer's yeast	0	+6.09°	+5.57°	—	—1.41°	—	—
	60	+4.09°	+4.08°	25.5	—2.23°	59	—
	104	+3.10°	+3.37°	26.3	—2.73°	56	—
	149	+2.10°	+2.68°	27.5	—3.35°	51	—
Sauterne yeast	0	+5.88°	+5.38°	—	—1.36°	—	—
	34	+3.62°	+3.75°	28	—1.99°	72	—
	60	+2.02°	+2.63°	29	—2.92°	60	—

Exp. 3. Selective fermentation by yeast weak in saccharase.

Washed pressed brewer's yeast was treated with 0.2 *N* sulphuric acid for one hour and its saccharase value substantially reduced. Another portion suffered similarly by treatment with 0.05 *N* sodium hydroxide. A third portion was steeped in water. Selective fermentations were performed with the three yeasts immediately after washing. The results appear in Table I, Exps. 8, 9, 10.

Exp. 4. Influence of saccharase on selective fermentation by zymin, prepared from brewer's yeast.

To 200 cc. of a solution of the mixed sugars were added 10 g. of zymin; after mixing the solution was divided into two equal portions and a saccharase preparation added to one, and the same volume of boiled preparation to the other. A drop of toluene was added to each, and fermentation allowed to proceed at 25°. After a suitable gas evolution had occurred, fermentation was arrested by clearing with alumina cream, boiling and clearing afresh. The residual solution was analysed as for the yeast fermentations, and finally fermented to completion with living yeasts and again analysed, the latter results being deducted from the previous ones. In Exp. A, zymin from lager brewery yeast was used, and the saccharase preparation was a crude autolysate of yeast, too old to contain any co-zymase. In Exp. B, top brewery yeast zymin and a purified saccharase obtained from yeast autolysate by adsorption with aluminium hydroxide (Willstätter and Kraut) and eluted with dilute ammonia.

Table III. *Selective fermentation by zymín with and without added saccharase.*

	Saccharase preparation		Sugars g. per 100 cc.	
			Glucose	Fructose
A. Lager yeast zymín	Crude yeast autolysate Control	Initial conc.	3.80	3.80
		Fermented by zymín	0.70	0.35
		Initial conc.	3.80	3.80
		Fermented by zymín	0.62	0.28
B. Top yeast zymín	Purified saccharase Control	Initial conc.	3.33	3.02
		Fermented by zymín	1.97	0.18
		Initial conc.	3.33	3.02
		Fermented by zymín	2.55	0.30

Exp. 5. Velocity of fermentation of glucose and fructose in separate solutions.

This was measured at 30° in the apparatus devised by Slator [1906]. The highest rate observed in the experiments with both brewer's and Sauterne yeasts was with fructose at 3.5 % in the fermenting mixture, and this was adopted as a standard in each case. The results, expressed in terms of these standards as 100 are given in Table IV. Autolysis rates have been deducted.

Table IV. *Relative rates of fermentation of glucose and fructose at 30°.*

	Sugar g. per 100 cc. of fermenting liquid	Glucose	Fructose
Brewer's yeast	0.20	51	31
	0.40	76	52
	1.00	89	90
	3.50	95	100
Sauterne yeast	0.20	—	43
	0.40	10	75
	0.50	15	79
	1.00	34	88
	3.50	54	100
	9.00	65	95
	10.50	71	87

Exp. 6. Selective fermentations of glucose and fructose at different temperatures.

In this experiment it is desirable to avoid either excessively fast or slow fermentations. In the former case the fermentation may be fast enough to compare with the rate of mutarotation of glucose, and in the latter, prolonged exposure to different temperatures may cause variations in the character of the yeast. Samples were withdrawn from the fermentations at intervals by rapid centrifugation followed by filtration. The procedure was as in Exp. 1, except that the reducing sugars were estimated by the Hagedorn-Jensen method as modified by Hanes, using the table of values furnished by Sobotka and Reiner [1930, 1]. In the case of fermentations with Sauterne yeast it was found desirable to start with a much higher proportion of glucose than of fructose, otherwise experimental errors are unduly large, and the appropriate juncture at which to withdraw a sample is difficult to judge. The results are

given in Table I, Exps. 11 to 20. The concentrations used in Nos. 18-20 were chosen with due regard to the results of Exp. 5.

Exp. 7. Mutarotation of glucose solution when partly fermented by yeast.

The experiments with glucose were performed by much the same procedure as was adopted by Willstätter and Sobotka [1922] except that the percentage of sugar fermented was determined by a separate withdrawal, and the addition of chloroform to arrest fermentation was omitted. About 200 g. of well-washed pressed yeast were added to an equal weight of 15 % glucose solution, and fermentation was allowed to proceed at 30° with frequent stirring. After a suitable time a few cc. of *N* acetic acid and a quantity of powdered ice were added and the liquid filtered rapidly through a large filter previously layered with alumina cream (free from salts, and neutral). The first runnings were rejected. The rotation could be read within two or three minutes of zero time, the latter being taken as the mean of the time of commencement and completion of filtration of the solution required to fill the observation tube. Fermentation was still proceeding in the funnel and was only arrested as the liquid passed through the filter. The acetic acid was added to ensure the attainment of a p_H of about 3.6 at which the rate of mutarotation is at a minimum. The fermentations with Sauterne yeast were performed at 20° and no ice added. It was found that less error was involved in this way, since when glucose is suddenly chilled a slow fall in rotation follows. The values of the constant $K_{\alpha/\beta}$ were calculated as by Willstätter and Sobotka, assuming $[\alpha]_D$ for α -glucose + 110°, for β -glucose + 19° and for the equilibrium solution + 52.6°.

Table V. *Mutarotation of glucose solutions partly fermented by yeast.*

% sugar fermented	Initial (extra- polated from $t_1, t_2, \dots$)	Polarimeter readings					$[\alpha]_D$ at time of arresting fermen- tation	$K_{\alpha/\beta}$	
		t_1	t_2	t_3	t_4	Final t_∞			
Brewer's yeast:									
28.7	9.67°	6 min. 9.73°	12 min. 9.77°	60 min. 9.88°	—	10.20°	+ 49.8°	1.53	
31.9	9.12°	3 min. 9.24°	5 min. 9.28°	60 min. 9.46°	—	9.74°	+ 49.2°	1.47	
38.6	8.20°	3 min. 8.28°	14 min. 8.38°	27 min. 8.44°	100 min. 8.58°	8.78°	+ 49.1°	1.38	
41.3	7.80°	3 min. 7.90°	14 min. 8.05°	—	107 min. 8.19°	8.40°	+ 48.8°	1.38	
Sauterne yeast:									
—	12.31°	4 min. 12.29°	45 min. 12.19°	100 min. 12.14°	145 min. 12.09°	11.99°	+ 54.0°	—	
—	11.60°	3 min. 11.58°	45 min. 11.52°	100 min. 11.50°	—	11.25°	+ 54.2°	—	
—	5.75°	4 min. 5.73°	48 min. 5.68°	—	—	5.56°	+ 54.4°	—	

Exp. 8. Mutarotation of fructose solution when partly fermented by yeasts.

The procedure was as for glucose, with the following differences. In working with fructose much greater speed of observation is necessary than with glucose owing to the greater rate of mutarotation. It is also necessary to keep the temperature absolutely constant throughout fermentation, filtration and polarimetric observations. The experiments were performed at laboratory temperature, about 10°. It was not possible to calculate the values of K since the proportions of the constituents of fructose solution at equilibrium are unknown. Should the latter information become available, the data here presented may be of possible use in this connection.

Table VI. *Mutarotation of fructose solutions partly fermented by yeast.*

% sugar fermented	Polarimeter readings						[α] _D ^{20°} at time of arresting fermen- tation
	Initial (extra- polated from $t_1, t_2, \dots$)	t_1	t_2	t_3	t_4	Final t_∞	
Brewer's yeast:							
13	9.37°	2 min. 9.30°	4 min. 9.27°	17 min. 9.10°	38 min. 8.98°	8.98°	- 97.1°
21	8.55°	2 min. 8.46°	5 min. 8.38°	21 min. 8.20°	40 min. 8.14°	8.14°	- 97.4°
25	8.12°	2 min. 8.05°	5 min. 7.99°	15 min. 7.86°	36 min. 7.79°	7.78°	- 97.1°
54	4.90°	2 min. 4.81°	4 min. 4.76°	23 min. 4.57°	50 min. 4.52°	4.51°	- 100.8°
56	5.06°	2 min. 4.94°	5 min. 4.84°	20 min. 4.66°	90 min. 4.64°	4.62°	- 101.8°
Sauterne yeast:							
44	6.00°	4 min. 6.05°	8 min. 6.08°	24 min. 6.12°	—	6.14°	- 90.8°
50	13.20°	3 min. 13.26°	—	42 min. 13.54°	—	13.60°	- 90.3°
50	8.56°	2 min. 8.64°	5 min. 8.73°	25 min. 8.93°	—	8.96°	- 89.0°
57	7.10°	2 min. 7.16°	3 min. 7.19°	28 min. 7.62°	75 min. 7.65°	7.72°	- 85.6°

NOTE ON THE CONFIGURATIONS OF GLUCOSE, MANNOSE AND FRUCTOSE.

In view of the foregoing hypothesis, it is interesting to compare the configurational formulae of the three hexoses for which yeasts of the brewer's yeast type are supposed to be specific. It will be seen that, apart from the reducing carbon atom and its attachments, the three hexoses, glucose, mannose and γ -fructose are identical in configuration. The suggestion put forward by E. F. Armstrong that the three fermentable hexoses owe this property to certain features of their configurations which they have in common, could be modified to fit the hypothesis now put forward. Normal fructose has little

in common with the other three hexoses. The formulae of Haworth have been adopted.

SUMMARY.

1. The factor $K_{G/F}$ for brewer's yeast was not appreciably influenced by the relative proportions of glucose and fructose in the original solution.
2. The factor was not influenced by modification of the saccharase activity of brewer's yeast by the method of Willstätter, Lowry and Schneider.
3. The saccharase of Sauterne yeast resembled that of brewer's yeast in that it was inhibited more strongly by fructose than by glucose.
4. The addition of saccharase to a mixture of glucose and fructose undergoing fermentation by zymine (brewer's) did not affect the selectivity.
5. The factor $K_{G/F}$ for brewer's yeast decreased with rise in temperature of fermentation, whilst that for Sauterne yeast increased.
6. Brewer's yeast ferments glucose faster than fructose in separate solutions when the concentration of sugar is less than 1 %. Sauterne yeast ferments fructose faster than glucose in separate solutions at all concentrations up to 10 %, but especially at low concentrations.
7. The mutarotation of partly fermented solutions of the sugars is for

glucose by brewer's yeast in the positive direction, glucose by Sauterne yeast in the negative, fructose by brewer's yeast in the positive, and fructose by Sauterne yeast in the negative direction.

8. The hypothesis is advanced that brewer's yeast and most yeasts are specific for that form of fructose which is present in small proportion, but which increases with temperature, possibly a γ -form, whereas Sauterne yeasts are specific for the normal form.

In conclusion I wish to express my thanks to Dr W. O. Kermack for the interest he has shown in this work, and for useful criticisms of the theories.

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