148. STUDIES ON THE ACETONE-BUTYL ALCOHOL FERMENTATION

1. NUTRITIONAL AND OTHER FACTORS INVOLVED IN THE PREPARATION OF ACTIVE SUSPENSIONS OF *CL. ACETOBUTYLICUM* (WEIZMANN)

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THIS work was undertaken in order to devise a technique for studying the acetone butyl alcohol fermentation by *Cl. acetobutylicum* (Weizmann).[1] As in the case of other organisms of this group fermentation has hitherto been carried out in growth experiments in which carbohydrate was fermented in the presence of protein and other material (maize meal, glucose in yeast water etc.) and the fermentation products estimated during or at the end of the fermentation period.

Obviously this procedure does not readily lend itself to the study of the intermediate fermentative processes which would be facilitated if washed suspensions of the organism could be used in manometers and other small-scale apparatus. The fact that much work has been done on fermentation by this group, notably by the Wisconsin school, without the employment of this technique has been noteworthy, but is readily explained by the many pitfalls encountered in the attempts here recorded.

This work falls into two parts: (1) the discovery of a growth medium in which the organism ferments glucose so as to give a good yield of acetone and butyl alcohol; (2) the obtaining from such a medium of active cells which when separated from it are still capable of fermenting glucose with similar results.

Methods and technique

The media used are described in the course of the paper. The organism was maintained in the spore condition on sand; 60 ml. 5% maize meal in tubes 325×28 mm. were inoculated from this and incubated for 24 hr.; the medium, freshly autoclaved and quickly cooled, was inoculated from these tubes in the proportion of 2 ml. inoculum to 100 ml. medium and the flask was then filled with H_2 or N_2; incubation was at 37°. At the end of the fermentation the culture was examined microscopically the film being stained by acid carbolfuchsin.

The analytical methods employed were as follows. Glucose was estimated by Hanes's [1929] modification of the method of Hagedorn & Jensen as described by Widdowson [1931]; acetic and butyric acids and ethyl and butyl alcohols by the methods of Friedemann [1938]; lactic acid by the method of Friedemann & Kendall [1929]; acetylmethylcarbinol by the method of Langlykke & Peterson [1937], pyruvic acid by the carboxylase method of Westerkamp [1933].

Acetone was estimated by the following method using a lactic acid apparatus of the type described by Friedemann *et al.* [1927] but having ground glass joints throughout and a sintered glass filter in place of the absorption tower of glass

[1] We are indebted to Dr A. C. Thaysen of the Chemical Research Dept., Teddington, for the strain used and also for much kind help in methods of cultivation.

beads. An aliquot of culture medium containing 0·6–1·2 mg. acetone is introduced into the distilling flask and diluted to 30–40 ml. The receiving flask contains 25 ml. $N/100$ iodine + 5 ml. N NaOH [Bell & Harrison, 1939]. The condenser water is turned on and a fairly rapid stream of air is drawn through the apparatus; the contents of the distilling flask are rapidly brought to boiling point and boiled for 10 min.; boiling is then discontinued. The residual I_2 is liberated by the addition of 7 ml. N H_2SO_4 and titrated against $N/100$ $Na_2S_2O_3$. Blank estimations are made and the acetone is calculated from the I_2 disappearing by means of the equation:

$$CH_3COCH_3 + 3I_2 + 4NaOH \rightarrow CHI_3 + CH_3COONa + 3NaI + 3H_2O.$$

The method gives 98% recovery from standard acetone solutions. None of the fermentation products formed by *Cl. acetobutylicum* interferes with the determination appreciably.

On 5% maize meal *Cl. acetobutylicum* gives a fermentation in which acetone and butyl alcohol amount to about 8·5% and 23·4% respectively of the starch fermented. 2% glucose in a boiling water extract of yeast gives variable results; frequently the yields of solvents are very low and the fermentation is mainly an 'acid' one, whilst sometimes solvents appear and the fermentation approaches

Table 1. *Products of the fermentation of 2% glucose in yeast water by a growing culture of* Cl. acetobutylicum

	Exp. 1		Exp. 2	
Substance	mg./ml.	% glucose fermented	mg./ml.	% glucose fermented
Glucose initial	21·2	—	21·8	—
Glucose final	11·0	—	1·6	—
Glucose fermented	10·2	—	20·2	—
Acetone	0·77	0·75	1·10	5·46
Ethyl alcohol	0·58	0·57	0·362	1·79
Butyl alcohol	0·925	9·1	3·25	16·1
Acetic acid	0·716	7·0	1·085	5·38
Butyric acid	1·993	19·5	1·44	7·13

'normal' (see Table 1). A growth medium was therefore sought upon which glucose was fermented so as to give yields approximating to those obtained from the carbohydrate of maize meal.

Search for a growth medium

The basal medium was approximately that of McDaniel *et al.* [1939] based on Speakman's salt mixture cited by these authors; it had the following composition: 0·05% K_2HPO_4; 0·05% KH_2PO_4; 0·02% $MgSO_4, 7H_2O$; 0·001% $MnSO_4, 3H_2O$; 0·001% $FeSO_4, 7H_2O$; 0·001% NaCl; 0·1% asparagine; a tryptic digest of casein equivalent to 0·25% casein. The above was autoclaved at double strength and the requisite volume of 15% glucose, slightly acidified and separately autoclaved, was added to it; small pieces of filter paper were also present. To this, the 'basal medium', certain growth factor preparations were added as set out below, and the whole was diluted so as to contain 2% glucose.

Growth factor preparations

(1) *Weizmann's autolysate of yeast* [Weizmann & Rosenfeld, 1937]. 100 g. baker's yeast are plasmolysed with 10 ml. ethyl acetate, kept at *p*H 7·0 with 10% Na_3PO_4, toluene added and incubated at 37° for 48 hr. It is then

centrifuged and the supernatant liquid diluted to 400 ml.; 1 ml. is therefore equivalent to 0·25 g. fresh yeast.

(2) *Liver autolysate.* 500 g. fresh liver are passed through a Latapie mincer; 100 ml. *M*/15 phosphate buffer *p*H 7·4 are added and the whole diluted to 1 l. Toluene is added and the suspension is incubated for 7–10 days at 37°. It is then centrifuged and the supernatant used.

(3) *Tryptic digest of liver.* 500 g. fresh ox liver are passed through a Latapie mincer and suspended in 1 l. distilled water; 100 ml. pancreatic extract and 100 ml. *M*/15 Na_2HPO_4 are added and the *p*H adjusted to 8·0; toluene is added and the whole incubated for 7 days at 37°, the *p*H being kept at 8·0; the digest is then filtered and the filtrate concentrated *in vacuo* to 500 ml.; 1 ml. is therefore equivalent to 1 g. fresh liver.

(4) *Tryptic digest of maize meal.* 500 g. maize meal are treated as described above for tryptic digest of liver.

Testing the media

The basal medium, without glucose, was made up in double strength; 15 ml. of this, together with the required amount of growth factor preparations, were made up to 30 ml. and autoclaved for 45 min. at 15 lb. pressure; 3 ml. 15% glucose autoclaved separately in acid solution were then added. This was inoculated with 0·5 ml. of a 24 hr. culture of *Cl. acetobutylicum* on 5% maize meal and incubated at 37° in H_2. Growth was estimated by inspection after 15 and 39 hr. and the acetone in the media then estimated. The results are given in Table 2. It was found that the basal medium alone was inadequate for regular growth or acetone production. Any of the four preparations added produced

Table 2. *Effect of certain additions to the basal medium on growth and acetone production by* Cl. acetobutylicum

Source of growth factor	Amount added % calculated as equiv. amount of fresh substance	Growth				Acetone mg./ml. 39 hr.
		15 hr.		39 hr.		
		(*a*)	(*b*)	(*a*)	(*b*)	
Yeast autolysate	10	+ + +	+ + +	+ + +	+ + +	0·1
	5	+ + +	+ + +	+ + +	+ + +	0·02
	1	+ +	+ +	+ + +	+ + +	0·55
	0·25	+	+	+ + +	+ + +	0·59
	0·10	+	+	+ + +	+ + +	0·53
	0·05	(+)	(+)	+ + +	+ + +	0·28
Yeast water	10	?	(+)	+ + +	+ + +	0·16
	5	+ + +	+ + +	+ + +	+ + +	0·24
	1	+ +	+ +	+ + +	+ + +	0·45
	0·25	+	+	+ + +	+ + +	0·12
	0·10	+	+	+ + +	+ + +	0·09
	0·05	(+)	(+)	+ + +	+ + +	0·11
Tryptic digest of liver	10	+ + +	+ + +	+ + +	+ + +	0·10
	5	+ + +	+ + +	+ + +	+ + +	0·15
	2·5	+ + +	+ + +	+ + +	+ + +	0·68
	1·0	+ +	+ +	+ + +	+ + +	0·80
	0·5	+	+	+ + +	+ + +	0·90
	0·25	(+)	(+)	+ + +	+ +	0·48
Tryptic digest of maize meal	5	(+)	+ +	+ + +	+ + +	0·32
	2·5	+	+ +	+ + +	+ + +	1·03
	1·25	+ +	+	+ + +	+ + +	0·05
	0·5	(+)	0	+ +	+ +	0·03
	0·25	0	0	(+)	0	0
	0·10	0	0	+	0	0

good growth and good acetone yields. In each case there was an optimum concentration above and below which the acetone yield fell off. It is evident that the four preparations tested contain a growth factor or factors which may or may not be identical with another substance necessary for acetone production.

Table 3. *Comparison of growth and acetone production on a medium containing 0·1% asparagine and 0·1% $(NH_4)_2HPO_4$ respectively*

15 ml. double strength basal medium +3 ml. 1·0% asparagine or 3 ml. 1% $(NH_4)_2HPO_4$ +growth factor preparation+water to 30 ml.+3 ml. 20% glucose (separately autoclaved in acid sol.) inoculated with 0·5 ml. 24 hr. maize meal culture, filled with H_2 and incubated at 37°.

Growth factor	Equiv. amount of fresh substance %	Asparagine		$(NH_4)_2HPO_4$	
		Growth	Acetone mg./ml.	Growth	Acetone mg./ml.
Yeast autolysate	5·0	+ + +	0·143	+ + +	None
	1·0	+ + +	0·05	+ + +	None*
	0·25	+ + +	0·725	+ +	Trace
	0·1	+	0·07	+ +	Trace
Tryptic digest of maize meal	5·0	+ + +	0·143	+ + +	Trace
	2·5	+ + +	1·58	+ + +	None
	1·0	+ + +	Trace	+ + +	None
	0·5	+ +	Trace	+ +	None
Tryptic digest of liver	5·0	+ +	Trace	+ +	Trace
	2·5	+ +	0·591	+ +	Trace*
	0·5	+ +	0·247	+ +	None
	0·25	+ +	0·133	+	None

* These cultures gave positive test for pyruvic acid.

The replacement of asparagine in the basal medium by an equivalent amount of $(NH_4)_2HPO_4$ had no perceptible effect on growth but almost eliminated the production of acetone, the traces still found being probably due to asparagine

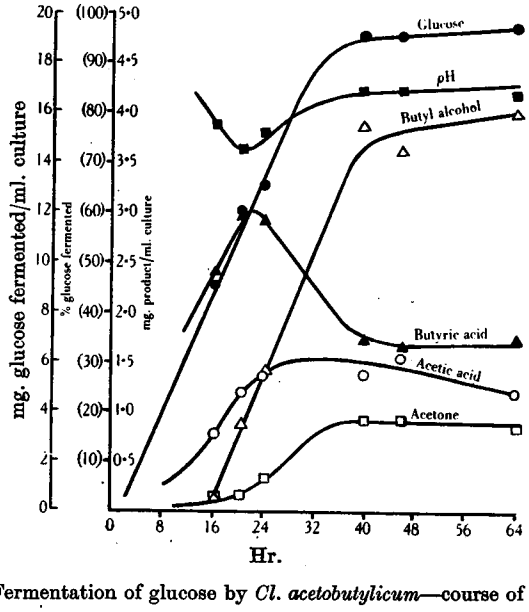

Fig. 1. Fermentation of glucose by *Cl. acetobutylicum*—course of reaction.

Table 4

Substrate	Medium	Product as % substrate (as sugar) fermented: Acetone	Butyl alcohol	Ethyl alcohol	Acetic acid	Butyric acid	Acetoin	CO_2	H_2	Formic acid	Lactic acid	Reference
1% glucose	0·5% peptone + salts	4·1	8·8	1·1	—	—	—	—	—	—	—	Johnson *et al.* [1931]
2% glucose	0·5% peptone + salts	8·3	20·5	2·5	—	—	—	—	—	—	—	Johnson *et al.* [1931]
4% glucose	0·5% peptone + salts	9·5	19·6	3·9	—	—	—	—	—	—	—	Johnson *et al.* [1931]
2·5% glucose	Yeast water	2·8	17·0	1·6	—	—	—	—	—	—	—	Langlykke *et al.* [1935]
2% glucose	Yeast water	3·4	20·2	—	10·3	10·5	—	51·7	1·75	Nil	Traces	Donker [1926]
4% maize meal	—	8·8	22·6	—	3·4	2·0	2·4	55·1	1·72	Nil	Traces	Donker [1926]
2% glucose	Yeast water	7·2	23·0	2·4	4·7	2·1	3·1	54·0	1·5	Traces	—	van der Lek [1930]
Maize meal* (industrial scale)	—	12·1	22·7	1·9	—	—	—	—	—	—	—	Speakman [1920]
6·5% maize meal* (1540 gallons of mash)	—	9·7	23·1	—	0·93	0·69	—	56·9	1·44	—	—	Reilly *et al.* [1920]
2% glucose	Tryptic digest of liver + salts + asparagine	7·9	23·8	Nil	5·73	6·75	—	—	—	—	—	Present authors
4·3% maize meal	—	8·5	23·4	0·45	2·2	1·44	4·1	—	—	—	Nil	Present authors
2% glucose in *M*/5 acetate	Tryptic digest of liver + salts + asparagine	14·2	21·2	0·2	—	—	—	—	—	—	—	Present authors
7·4% maize meal	—	14·2	26·4	3·0	0·71	1·55	—	—	—	0·08	—	Stiles *et al.* [1929]

* Yields recalculated from authors' figures to refer to % glucose fermented.

Table 5. *Effect of various substances on the production of gas from glucose by washed suspensions of* Cl. acetobutylicum

	Glucose	Pyruvate	Glucose and Pyruvate	Filtered culture	Cell washing	Yeast autolysate	Glyceraldehyde	Glycerophosphate	Acetaldehyde	Formate	Asparagine	Glyceric acid	Methylene blue
Q_{gas}*	<1	24	34	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1

* Q_{gas} = μl. total gas calculated as H_2/hr. and mg. dry wt. cells.

introduced with the growth factors,[1] see Table 3. In this connexion a possible parallel is found in the work of Tatum *et al.* [1935] and of Brown *et al.* [1938] who found in the case of the allied organism *Cl. butylicum* that asparagine added to the growth-fermentation maize-meal medium raised the production of neutral solvents, chiefly butyl alcohol, from 0·8 % to 19·9 % of the carbohydrate fermented. *Cl. acetobutylicum* shows no such effect with maize meal; possibly the latter organism possesses sufficient proteolytic activity to liberate asparagine from the maize proteins.

The medium finally adopted for growing the organism was the basal medium + tryptic digest of liver equivalent to 2·5 % fresh liver tissue. Growth on this medium produces a satisfactory amount of acetone and butyl alcohol and the course of the fermentation agreed with that obtained by other workers on this and related organisms (see Fig. 1 and Table 4).

The use of washed suspensions

In order to study the course of the fermentation it was desired to use manometric and other techniques involving washed suspensions of the organism. Great difficulty was encountered here owing to the fact that cells from an actively fermenting culture, when washed in *M*/15 phosphate buffer at *p*H 6·0, are generally inactive when put into glucose and buffer. This is the case whether the criterion of activity be reduction of methylene blue by glucose, gas production from or disappearance of glucose. Generally reduction of methylene blue was more active than gas production but the activity fell off rapidly. Finally it was decided to concentrate on obtaining a suspending solution in which the cells should show vigorous and sustained evolution of H_2 and CO_2 from glucose and pyruvate when tested in a manometer.

Warburg manometers were used each containing 1 ml. *M*/15 phosphate buffer *p*H 5·0; 1 ml. cell suspension (equivalent to 10 mg. dry wt./ml.) in the main vessel and 0·2 ml. *M*/10 glucose or *M*/15 pyruvate in one side bulb; the manometers were gassed with H_2 and shaken at 37°. Little or no gas production having been obtained in 30 experiments it was thought that some intermediate product might be needed as a 'starter' substance. The experiments were therefore repeated with the following substances in the second side bulb of the manometer; 0·2 ml. of one of the following: *M*/10 pyruvate; *M*/10 glyceraldehyde; *M*/10 Na glycerophosphate, *M*/10 acetaldehyde; *M*/10 Na formate; *M*/10 asparagine; 0·5 ml. filtered culture medium after growth and fermentation were complete; 0·5 ml. washings from cell suspensions; 0·5 ml. yeast autolysate; 0·5 ml. 0·1 % methylene blue. The results are given in Table 5; none of these substances was effective.

It was clear that in the culture medium the cells were active but that they lost their activity in the process of centrifuging, washing and resuspending in buffer. This was confirmed by centrifuging the culture and suspending the cells in the original culture fluid; the result was termed the 'concentrated culture'; 1 ml. of this used in a manometer gave a gas evolution of 350 μl. during 1 hr. owing to residual fermentable material. When this was over fresh glucose was tipped in from the side cup and a rapid gas evolution was obtained ($Q_{gas} = 258$, calc. as H_2). No gas evolution was obtained from a washed suspension of the same organisms.

The effect of suspending the centrifuged organisms in phosphate buffer without washing was then tried; this suspension produced no gas but was active

[1] In some preparations this may be sufficient to supply all the asparagine necessary.

in reducing methylene blue with glucose (see Table 6). A further increase in activity was obtained by de-oxygenating the suspending fluid by boiling, the $Q_{\text{methylene blue, glucose}}$ rising in one experiment from 33 to 216.

Table 6. *Effect of washing on the reduction of methylene blue by glucose and pyruvate using suspensions of* Cl. acetobutylicum

Each Thunberg tube contained 1 ml. phosphate buffer *p*H 5·0, 1 ml. *M*/10 glucose or *M*/10 pyruvate, 0·25 ml. 0·1% methylene blue. Temp. 37°.

	$Q_{\text{methylene blue}}$		
Substrate	Conc. culture	Unwashed suspension	Washed suspension
None	65	5	<1
Glucose	—	97	16
Pyruvate	—	70	37

The influence of various constituents of the culture medium in maintaining the activity of the culture was then tried, the activity of the 'concentrated culture' being the standard of comparison; Fig. 2 shows that all the constituents

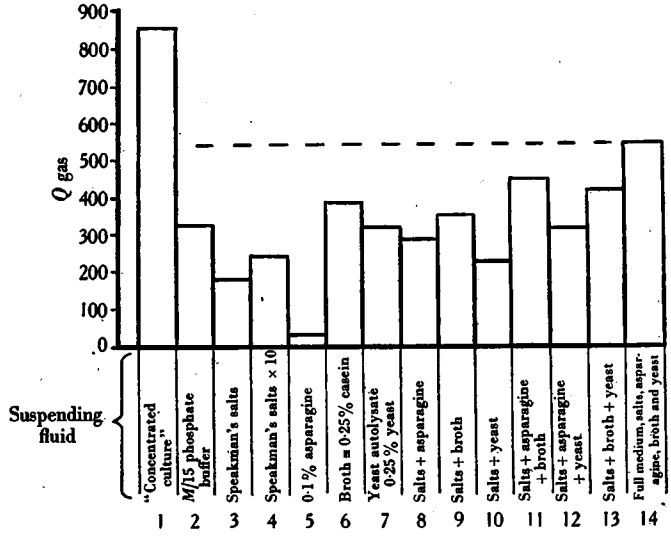

Fig. 2. Effect of suspending solution on unwashed cells as measured by gas production.

of the original medium contribute to the activity of the cells and that when the cells are resuspended in the original medium they retain 63% of their gas-producing activity. The gas production from pyruvate is less dependent on the suspending fluid, suspensions in solutions 2, 3, 8, 9, 10 and 14 giving a Q_{gas} between 222 and 266. In all subsequent experiments the cells were suspended in freshly boiled basal medium + yeast autolysate equivalent to 0·25% fresh yeast; the appropriate substrate being added from the side cup of the manometer vessel or as the experiment demanded. This medium is referred to as the Y.A. medium. The gas production from this medium without substrate is negligible and inactive suspensions are seldom obtained, provided that the suspension is used within a few minutes of its preparation as, even with these precautions,

decay is very rapid; for example, in the case of glucose the Q_{gas} fell from 400 to 18 in $2\frac{3}{4}$ hr.; that of pyruvate from 283 to 15 in 5 hr. A suitable growth medium and suspending fluid having been arrived at, preliminary experiments were now possible.

The pH optimum for the fermentation of glucose and pyruvate

The effect of *p*H on glucose fermentation was determined by three methods: (1) total gas production; (2) production of H_2; (3) disappearance of glucose. (1) and (2) were determined manometrically and (3) by the estimation of glucose. A preliminary difficulty was a choice of buffers to cover the range *p*H 3·5 to 7·5; the only single buffer available was McIlvaine's citrate-phosphate mixture [1921], but this inhibited the fermentation of glucose. Acetate buffers cover the range *p*H 3·5–6·0 but it was feared that, as acetic acid is a product of fermentation, this might have a disturbing influence on the fermentation; on testing the gas evolution at *p*H 5·0 in the presence of acetate and phosphate buffers respectively it was found that none occurred in the absence of glucose (or pyruvate) and that in the presence of substrate the rate with both buffers was identical; accordingly *M*/10 acetate was used for the range *p*H 3·5–6·0 and phosphate for the range *p*H 6·0–8·0.

For the manometric experiments each vessel contained 1·0 ml. cell suspension (about 5 mg. dry wt./ml.), and 1·8 ml. buffer (*M*/10 acetate or *M*/15 phosphate) in the main cup; 0·2 ml. *M*/10 glucose or pyruvate in the side bulb. In the case

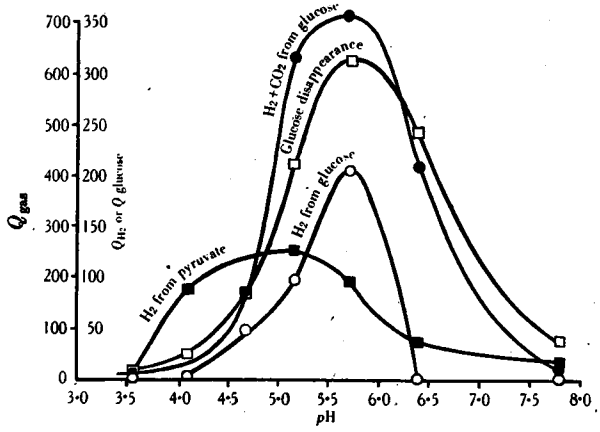

Fig. 3. *p*H-activity curves for fermentation of glucose and pyruvic acid by *Cl. acetobutylicum* (Weizmann).

of H_2 evolution 0·2 ml. 20% NaOH and pleated filter paper were used in the centre cup; the vessels were gassed with H_2; the substrates were tipped into the main compartment after equilibration and the initial velocity of gas evolution over a period of 10 min. taken. The results are shown in Fig. 3.

Glucose disappearance was measured as follows: Krebs vessels were set up each containing 18 ml. buffer, 2 ml. *M*/10 glucose and 10 ml. cell suspension. H_2 was passed through the vessels for 5 min. and they were incubated at 38°. Samples were removed at intervals and glucose estimated. The glucose disappearing was plotted against time and the initial rate calculated; this was expressed as $Q_{glucose}$ (μl. glucose used/mg. dry wt. organism hr.; 1 m.-mol. = 22,400 μl.).

Fig. 3 shows that the optimum fermentation rate for glucose occurs at *p*H 5·7 irrespective of whether total gas evolution, H_2 evolution or glucose utilization is taken as the criterion. The optimum rate for pyruvate fermentation as measured by H_2 production was at *p*H 5·1.

Character of the fermentations of glucose and pyruvate by washed suspensions in Y.A. medium

In order to determine whether the fermentation by washed suspensions in Y.A. medium was an 'acid' or a 'normal' one the following products were estimated: acetone, butyl alcohol, acetic and butyric acids, acetoin, lactic acid, residual substrate, CO_2 and H_2.

The organisms were grown in the usual way, centrifuged and suspended in Y.A. medium; the fermentations were done in Krebs vessels; H_2 and CO_2 were measured manometrically each manometer containing 1/40th of the volume

Table 7. *Products of fermentation of glucose by cell suspensions of* Cl. acetobutylicum

Krebs vessels contained 30 ml. *M*/15 phosphate buffer *p*H 5·12, 10 ml. *M*/10 glucose, 20 ml. cell suspension 9·5 mg. dry wt./ml.; gassed with H_2; temp. 37°. The fermentation was complete in 4 hr.

	Product mg.	Product as % glucose fermented	C in product mg.	H in product mg.	O in product mg.
Initial glucose	360	—	—	—	—
Final glucose	33·3	—	—	—	—
Glucose fermented	326·7	—	130·7	21·8	174
H_2	6·96	2·13	—	6·96	—
CO_2	156·5	48	42·7	—	113·8
Acetic acid	36·0	11·0	14·4	2·24	19·2
Butyric acid	107·6	32·9	58·7	9·79	33·8
Acetone	4·9	1·5	3·0	0·57	1·4
Butyl alcohol	None	—	—	—	—
Ethyl alcohol	„	—	—	—	—
Pyruvic acid	„	—	—	—	—
Lactic acid	„	—	—	—	—
Acetoin	„	—	—	—	—
Total C, H and O in products			118·8	19·5	168·2

Table 8. *Products of fermentation of pyruvic acid by cell suspensions of* Cl. acetobutylicum

Krebs vessels contained 20 ml. *M*/15 phosphate buffer *p*H 4·86, 20 ml. cell suspension 23 mg. dry wt./ml.; 20 ml. *M*/20 pyruvate; gassed with H_2; temp. 37°. Fermentation complete in 4 hr.

	Total product mg.	Product as % pyruvate fermented	C in product mg.	H in product mg.	O in product mg.
Initial pyruvic acid	155·5	—	—	—	—
Final pyruvic acid	None	—	—	—	—
Pyruvic acid fermented	155·5	—	63·62	7·06	85·00
H_2	2·24	1·44	—	2·24	—
CO_2	60·3	38·8	16·46	—	43·85
Acetic acid	80·2	51·6	32·08	5·31	42·70
Butyric acid	19·15	12·3	10·41	1·74	6·97
Acetone	None	—	—	—	—
Ethyl alcohol	„	—	—	—	—
Butyl alcohol	„	—	—	—	—
Acetoin	„	—	—	—	—
Lactic acid	„	—	—	—	—
Total C, H or O			59·95	9·29	93·52

used in the Krebs vessel; the quantities used are given in Tables 7 and 8; the manometric experiment was run concurrently with the main experiment and served also to show when the reaction reached a standstill. 30 min. after the gas evolution in the manometers had ceased the contents of the Krebs vessels were centrifuged and the deposit of organisms washed once; the supernatant and washings were made up to 70 ml. and analysed. It is clear from Tables 7 and 8 that the fermentation occurring is essentially an acid one, acetone being absent in the case of pyruvate and present in low amounts only from glucose. (In our experience the production of acetone and the alcohols from glucose are so closely related that for exploratory experiments the former only need be estimated.) The cause of the absence of solvents was next investigated.

The effect of the age of the culture on the character of the fermentation

A fermentation of glucose in 5 l. of the usual glucose-liver medium was carried out. The 'age' of this culture was checked by glucose determinations, *p*H measurements and acetone determinations. Samples were withdrawn at intervals and used for the preparation of suspensions in Y.A. medium. The fermenting power of these suspensions was tested as follows: 7 ml. cell suspension, 4·2 ml. 2 *M* acetate buffer at *p*H 5·0, 1 ml. *M* glucose, 1·4 ml. 1·5 % asparagine and 7·4 ml. water were put into a Thunberg tube modified so as to permit of gassing with H_2. After 20 hr. glucose disappearance and acetone production were measured. The results are given in Tables 9 and 10.

Table 9. *Effect of age of culture on acetone produced* (*a*) *in the culture medium,* (*b*) *by cell suspensions prepared from it*

Age of culture hr.	Growth mg./ml. dry wt.	% glucose fermented	Acetone in culture mg./ml.	Acetone formed by cell suspensions mg./ml.
6	0·291	5	None	None
8	0·965	11·8	None	None
10	1·30	25·3	0·038	0·09
12	1·34	33·9	0·09	0·154
14	1·30	38·0	0·123	0·190
17	1·24	40·2	0·163	0·111
22	1·35	41·6	0·181	0·091
24	1·085	44·8	0·193	0·251
43	0·695	71·5	0·707	0·082

Table 10. *Effect of the age of the culture on the activity of the suspensions of* Cl. acetobutylicum

Age of culture hr.	Growth mg./ml. dry wt.	% glucose fermented	Acetone in culture mg./ml.	Acetone formed by cell suspension mg./ml.
13	1·36	34·0	0·137	0·594
16	1·40	47·5	0·298	—
19	1·38	56·7	0·493	2·10
22	1·32	65·8	0·653	2·09
25	1·19	74·9	1·106	1·91
40	—	95·4	1·15	0·051

In Table 9 it is seen that up to 8 hr. no acetone occurred in the parent culture neither was acetone produced by the corresponding suspensions. From 10 hr. onwards acetone appears in the parent culture and is also formed by the suspensions. The activity of the suspension in respect of acetone production increases with increasing amounts of acetone in the parent culture up to a maximum, after

which it remains fairly constant for some hours. Without emphasizing the quantitative aspect of these experiments one deduces that in the early stage of the fermentation some essential part of the enzyme complex necessary for acetone production is absent; not until acetone is present in the parent culture is the corresponding suspension capable of producing it. Hence, when investigating acetone production by cell suspensions, it is necessary to harvest the cells after the appearance of acetone in the culture medium; such suspensions will hereafter be referred to as 'ripe' suspensions.

Fermentation by suspensions of ripe organisms

The organisms were grown on the usual medium and after the appearance of acetone the cells were harvested and suspended in Y.A. medium. The suspensions were incubated with $M/10$ glucose in an atmosphere of H_2 and analyses made

Table 11. *Fermentation of glucose in Y.A. medium*

Krebs vessel contained 8 ml. *M* glucose, 22 ml. Y.A. medium and 50 ml. cell suspension (26·4 mg./ml. dry wt.). The medium was inoculated as usual and gassed with H_2. Samples were withdrawn at 6 hr. and 22 hr. for analysis and determination of *p*H.

	Initial	6 hr.	22 hr.	Products as % glucose fermented
Glucose, mg./ml.	20·0	1·23	1·07	—
*p*H	5·97	4·40	4·47	—
Acetone, mg./ml.	—	0·87	0·81	4·5
Butyl alcohol, mg./ml.	—	3·5	3·6	18·9
Butyric acid, mg./ml.	—	—	2·57	13·6
Acetic acid, mg./ml.	—	—	1·47	7·8

after 6 and 22 hr. The results are given in Table 11. This shows that a good production of solvents can be obtained by suspensions of the organisms if harvested at the right time and suspended in an adequate medium.

Summary

1. The fermentation of carbohydrate by *Cl. acetobutylicum* depends on the presence of a number of factors besides the organism and the carbohydrate.

2. On 5% maize meal the organism grows readily and ferments the carbohydrate of the maize, producing CO_2, H_2, acetic and butyric acids, ethyl and butyl alcohols and acetone ('normal fermentation'). On 2% glucose and yeast water the yields of alcohols and acetone are very low ('acid fermentation').

3. In order to convert an acid fermentation into a normal fermentation certain additions are necessary; these have not yet been isolated but are present in liver, maize and yeast and can be extracted from these by suitable procedures. There is an optimal amount of each of these extracts above and below which the yield of acetone falls off.

4. Cells grown on a basal medium + liver extract give good growth and solvent production but when spun off and washed are totally inactive.

5. A suspending fluid in which the centrifuged cells retain their fermentative activity was at length found by the use of which the course of the fermentations of glucose and pyruvate have been studied.

6. The character of the fermentation depends on the age of the culture from which the cells are harvested; those obtained in the early stages of the fermentation produce no acetone in subsequent fermentations; only if harvested after the appearance of acetone in the parent culture are cells obtained which later produce a normal fermentation. Such organisms are termed 'ripe'.

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