THE METABOLISM OF GLUCOSE BY ASHBYA GOSSYPII

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Considerable interest has been centered on *Ashbya gossypii* recently because of its ability to synthesize large quantities of riboflavin. The work of Wickerham, Flickinger, and Johnson (1946) called attention to the potential industrial significance of certain strains for the manufacture of riboflavin. During the course of studies on the synthesis of riboflavin it became of importance to learn something about the physiological behavior of the organism. This communication is concerned with the metabolism of glucose by a culture of *A. gossypii.*

METHODS

The organism is a filamentous yeast that is predominantly aerobic in growth characteristics, and the most suitable mycelial preparations for study were obtained by culturing the organism in liquid media with aeration. The culture, obtained from the collection of the Northern Regional Laboratory,[1] was carried on an agar medium consisting of 1 per cent glucose, 0.5 per cent Difco peptone, and 0.3 per cent Difco yeast autolyzate. A medium of this composition, without the addition of agar, was used to obtain mycelial suspensions for manometric and fermentation studies. Mycelial suspensions cultured for 24, 48, or 72 hours were obtained by incubating at 28 C on a reciprocating shaker run at 54 cycles per minute. The suspensions were dispersed by being placed in a Waring blender for 15 to 30 seconds and subsequently being washed at least four times with distilled water on the centrifuge. For manometric studies 5 to 10 mg of dry cell tissue per respirometer vessel were suspended in 0.1 M phosphate buffer at pH 6.8 with a 0.02 M substrate concentration. Measurements were made with a Barcroft-Warburg respirometer at 30 C.

In the serial analysis of aerobic fermentations, the carbon dioxide was collected in 200 ml of 1 N sodium hydroxide in a Drechsel absorption bottle. The alkali was renewed at the time each sample was removed, and the carbon dioxide was determined gravimetrically on an aliquot portion of the alkali according to the procedure of Mickelson and Werkman (1939). The fermented medium was analyzed for fermentation products by the techniques described in the same publication. Sugar analyses were made according to the method of Underkofler, Guymon, Rayman, and Fulmer (1943). Before analysis, samples were deproteinated as follows: to 1 to 5 ml of fermented medium were added 0.5 ml saturated lead acetate and a few crystals of solid oxalic acid. The sample was diluted to 10 ml and an aliquot portion used for analysis after sedimentation of the precipitate. Pyruvic acid was determined on an ether-extracted sample of the fermented liquor by the iodoform reaction. It was also estimated by the colorimetric method

[1] Appreciation is expressed to Dr. L. J. Wickerham for the culture of *Ashbya gossypii.*

of Friedemann and Haugen (1943) with the modifications of Lichstein and Umbreit (1947), the two values agreeing closely. Riboflavin was determined fluorometrically with a Coleman model 11 spectrophotometer. Fermentations were conducted at 30 C and maintained under aerobic conditions by sparging filtered air through the medium with a sintered glass disk.

RESULTS

Aerobic metabolism. It was difficult to obtain tissue suspensions with low endogenous oxygen uptake values. With the most suitable preparations the endogenous respiration was usually about one-third of that found in the presence of substrate. If the mycelium was harvested, resuspended in buffer, and aerated for 24 hours, the endogenous activity was reduced but the activity on a specific

TABLE 1

Influence of the age of cells at the time of harvesting on the ability to oxidize various substrates

(Substrates, 0.02 M; phosphate, 0.1 M; pH 6.8; 30 C; 120 min; 5 mg dry mycelium per flask)

SUBSTRATES	MICROLITERS OF O_2 CONSUMED					
	24 hr		48 hr		72 hr	
None	193	*	106	*	83	*
Glucose	514	321	304	198	202	119
Sucrose	490	297	279	173	210	127
Maltose	231	38	165	59	181	99
Lactose	191	−2	100	−6	78	−5
Pyruvate	270	77	132	26	89	6
Acetate	249	56	198	92	156	73
Ethanol	312	119	189	83	117	34

* O_2 uptake corrected for endogenous respiration.

substrate was also lowered significantly. Mycelial suspensions from cultures grown in the medium and under the conditions described above were used. If they were lyophilized or frozen, respiratory activity was lost completely and it was not restored upon the addition of boiled yeast juice and mineral salts. No success has yet been attained with acetone-treated mycelium.

In table 1 are shown the relative abilities of suspensions of cells to oxidize various substrates when cultured for 24, 48, and 72 hours. The values shown are total microliters of oxygen consumed per flask per 120 minutes, as well as the same values with the endogenous O_2 uptake substracted. Of the sugars tested, glucose and sucrose were most readily oxidized, maltose being oxidized more slowly, and lactose was not attacked. Ethyl alcohol and pyruvic and acetic acids were also oxidized. In general, the 24-hour cells were more active than the 48-hour cells and they in turn more active than the 72-hour cells. There was an exception in the case of maltose, with which this order was reversed; in the oxidation of acetic acid the 48-hour suspension of cells was most active.

A serial analysis of an aerobic dissimilation of glucose was made in which the unfermented glucose, carbon dioxide (formation), and pH were determined on each sample. The fermentation was conducted in 300 ml of a medium consisting of 1.5 per cent glucose, 0.5 per cent Difco peptone, and 0.3 per cent Difco yeast extract. The medium was inoculated with a washed suspension of mycelium from a 24-hour culture in 25 ml of the foregoing medium and incubated during the course of the experiment at 30 C. The aeration rate was 0.2 of a liter of air per liter of medium per minute.

On examination of figure 1, the following findings are apparent. During the first 72 hours there were a rapid utilization of glucose and a parallel drop in pH

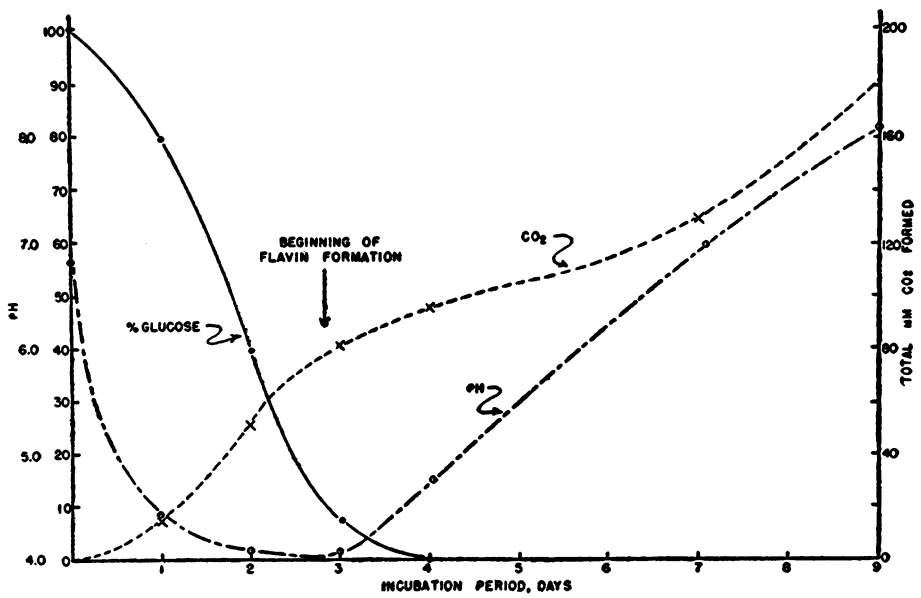

Figure 1. Serial analysis of a glucose fermentation by *Ashbya gossypii*. Medium: 1.5 per cent glucose; 0.5 per cent Difco peptone; 0.3 per cent yeast autolyzate. Temperature: 30 C. Aeration: 0.2 L/L/min.

of the medium from 6.8 to 4.2. When the glucose was exhausted, synthesis of riboflavin began, and there was a gradual rise in pH during the remainder of the incubation period until the pH reached 8.3. Carbon dioxide production continued throughout the incubation period until at the end it accounted for about 95 per cent of the sugar consumed.

It was possible that the rather high recovery of carbon may have been due to the oxidation of products resulting from the decomposition of the peptone and yeast extract. In an attempt to avoid this difficulty a second experiment was conducted in a medium of 2 per cent glucose, 0.3 per cent ammonium sulfate, 0.03 per cent peptone, and 0.02 per cent yeast extract. Though growth was not luxuriant, 77 per cent of the glucose disappeared during an incubation period of 10 days. From 26.4 mM of glucose consumed, 114.5 mM of carbon dioxide (72

per cent of the carbon) and 1.44 mM of pyruvic acid (3.36 per cent of the carbon) were formed. Although the recovery of carbon in this experiment was not complete, it is interesting that some unfermented pyruvic acid was present. No volatile acid, ethyl alcohol, or nonvolatile acid other than pyruvic was found.

It is reasonable that the ability to oxidize acetate (table 1) would become more pronounced in the later stages of the incubation period. Presumably it is one of the final products of carbohydrate oxidation. The necessity for oxidizing the sugars, pyruvate, and ethyl alcohol would arise earlier in the culture than the necessity of oxidizing acetate.

A second experiment was set up in 1,000 ml of a medium consisting of 1.5 per cent glucose, 0.5 per cent Difco peptone, and 0.3 per cent yeast autolyzate,[2] and 100-ml samples were withdrawn during the course of the fermentation. A complete analysis for all of the products formed during aerobic dissimilation was made on each sample in order that some idea of the intermediate products could be ascertained. Analyses were made for volatile acids, lactic, pyruvic, and succinic acids, acetylmethylcarbinol, 2,3-butylene glycol, and ethyl alcohol. Only ethyl alcohol and acetic acid in addition to carbon dioxide were found. A trace of pyruvic acid was detected in the 24-hour sample by the nitroprusside color test.

The fermentation was incubated at 30 C and aerated at a rate of 0.3 to 0.4 of a liter of air per liter of medium per minute. This may account for the fact that the changes occurred more rapidly than in the first experiment. In general the pH, glucose consumption, and carbon dioxide production curves are similar for both experiments (figure 2). (The numbers of the vertical axis refer to acetic acid, ethyl alcohol, riboflavin, and percentage of glucose.) Analysis for other products revealed the presence of small amounts of ethyl alcohol and traces of acetic acid. In the 24-hour and 48-hour samples, the yield of alcohol and carbon dioxide approached that of a typical alcoholic fermentation, i.e., 2 moles of each per mole of sugar fermented. After the disappearance of the sugar, oxidation of the alcohol to carbon dioxide resulted. Alcohol analyses were made in each case on the samples of fermentation liquor and on the 200-ml portion of alkali used for carbon dioxide absorption. Approximately one-half of the alcohol recovered was in the contents of the alkali absorption bottle. It is probable that a small amount escaped absorption under the conditions used. Judging from the carbon recovery value, however, the loss was not great. No nonvolatile acids or neutral nonvolatile products were formed. The data obtained in this experiment are summarized in table 2. The acetic acid resulting from the oxidation of the alcohol and the volatile acids formed by fermentation were identified by their partition constants according to the method of Osborn, Wood, and Werkman (1933).

The data from manometric experiments (table 4) suggest that in the absence of oxygen *A. gossypii* gives a typical alcoholic fermentation. The results obtained by serial analysis of an aerobic dissimilation also indicate that the primary pathway of glucose decomposition is that of a typical yeast fermentation.

[2] National Pharmaceutical Products Corporation, Crystal Lake, Illinois.

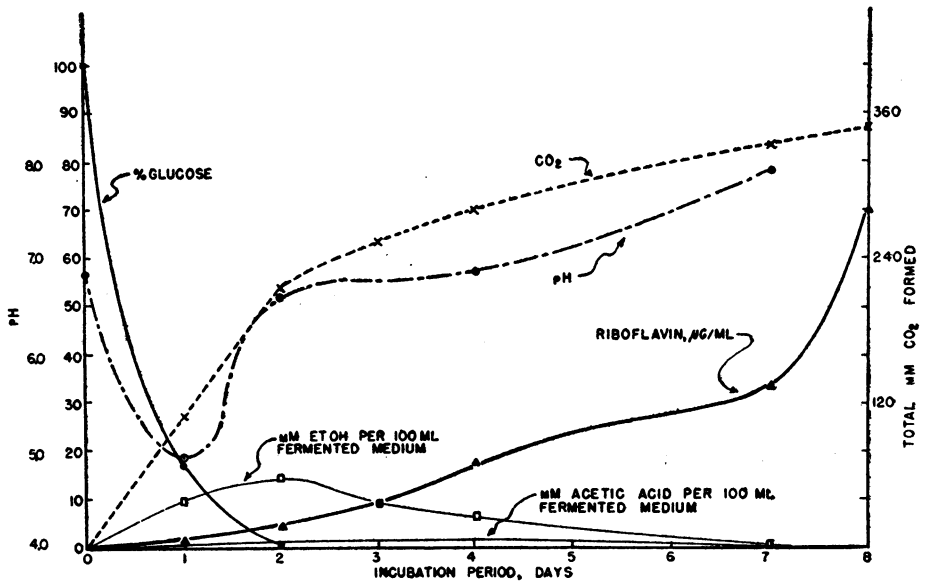

Figure 2. Serial analysis of a glucose fermentation by *Ashbya gossypii*. Medium: 1.5 per cent glucose; 0.5 per cent Difco peptone; 0.3 per cent yeast autolyzate. Temperature: 30 C. Aeration: 0.3 L/L/min.

TABLE 2

Serial analysis of glucose fermentation by Ashbya gossypii

(Medium: 1.5 per cent glucose, 0.5 per cent Difco peptone, 0.3 per cent yeast autolyzate)

HOURS	PER CENT OF GLUCOSE FERMENTED	TOTAL mM CO_2	GLUCOSE CONSUMED, mM PER 100 ML FERMENTED MEDIUM	PRODUCTS IN mM PER 100 ML FERMENTED MEDIUM			RIBOFLAVIN
				Ethyl alcohol	Acetic acid	Carbon dioxide	
							μg/ml
24	85.3	106.6	7.11	9.77	0.55	10.6	0.06
48	100	213.9	8.15	14.80	0.27	12.0	4.7
72	100	251.9	8.15	9.97	0.58	4.75	14.9
96	100	278.9	8.15	6.06	0.80	3.86	17.6
168	100	335.9	8.15	—	0.58	9.50	33.7
Final (500 ml).....	100	343.7	8.15		1.04(×5)	1.56	70.5
Total mM products		343.7		40.60	7.98		
Total mM carbon		343.7		81.20	15.96		
Total mM carbon recovered		440.86					
Carbon in glucose fermented mM		489.0					
Carbon recovery %		92.0					

The effect of several inhibitors on the aerobic metabolism of glucose is shown in table 3. Cyanide, 0.0001 M, completely inhibited all respiration, both glucose

oxidation and the endogenous oxygen uptake. A mixture of cyanide and KOH was used in the alkali well to prevent distillation of HCN from the main compartment into the alkali. With 0.01 M iodoacetic acid, only slight inhibition of endogenous respiration resulted, but inhibition of glucose oxidation was complete. Sodium fluoride in concentrations of from 0.01 M to 0.075 M completely inhibited glucose oxidation and reduced the endogenous respiration 46 to 82 per cent. Each inhibitor was tested against mycelial suspensions from different cultures that varied somewhat in activity.

Observations on the synthesis of riboflavin. The medium used in fermentation experiments was not suitable for synthesis of high yields of riboflavin. Under more suitable conditions, yields of riboflavin in excess of 1,000 micrograms per

TABLE 3

Effect of sodium fluoride, cyanide, and iodoacetate on the oxidation of glucose

(Glucose, 0.02 M; phosphate, 0.1 M, pH 6.8; temperature, 30 C; 180 min)

INHIBITOR	μL O_2 CONSUMED IN EXCESS OF CONTROL	PER CENT INHIBITION
None	268	0
0.01 M NaF	0	100
None	149	0
0.01 M iodoacetate	0	100
0.005 M iodoacetate	7	95
0.001 M iodoacetate	17	89
None	100	0
0.002 M cyanide	0	100
0.0002 M cyanide	0	100
0.0001 M cyanide	0	100

ml have been obtained. On the basis of these experiments, however, some observations can be made relative to riboflavin synthesis.

Riboflavin synthesis does not occur, in our experience, during the early stages of the fermentation when the sugar is being rapidly consumed and the pH is low. After the sugar has disappeared and the pH begins to rise, flavin formation begins and increases as the culture becomes older. In the early stages of the culture the mycelial growth is usually in the form of pellets. As culturing continues the volume of mycelium decreases, the pellets disintegrate, and the mycelium also begins to disintegrate until in the final stages only single cells and fragments remain. It appears that the synthesis of riboflavin occurs after maximum growth of the fungus has been reached and that the formation of flavin is in some way related to the transformation of cellular constituents synthesized by the growing mycelium rather than a direct synthesis from compounds added to the medium. However, the composition of the medium has a very important bearing on the ability of the culture to synthesize riboflavin. On some media good growth and sugar consumption occur but no riboflavin is synthesized.

Anaerobic metabolism. Though *Ashbya gossypii* has primarily an aerobic metabolism, it is able also to cause an anaerobic fermentation of glucose. Table 4 shows the results of reactions in an atmosphere of nitrogen with glucose as a substrate. The cells were grown aerobically in the medium previously described. The mycelium readily attacked glucose anaerobically, forming carbon dioxide, and there was practically no endogenous formation of carbon dioxide. When a definite quantity of glucose was added and the reaction allowed to go to completion in phosphate buffer, approximately 2 moles of carbon dioxide resulted from each mole of glucose consumed (table 4, *B*), which approaches the theoretical yield for an alcohol dissimilation. In the presence of bicarbonate, there was less carbon dioxide formed than in the presence of phosphate buffer.

TABLE 4

Anaerobic decomposition of glucose by A. gossypii

(Temperature, 30 C; atmosphere, N_2; time, 240 min)

A. Glucose, 0.02 M; $NaHCO_3$, 0.005 M; Phosphate, 0.1 M, pH 7.0

	CO_2 FORMED μL/FLASK
0.005 $NaHCO_3$	
Glucose	578
Endogenous	21
0.1 M phosphate	
Glucose	828
Endogenous	9

B

MICROMOLES GLUCOSE CONSUMED	MICROMOLES CO_2 PRODUCED	% OF THEORETICAL FOR ALCOHOLIC FERMENTATION
0.005 M $NaHCO_3$		
9.4	13.2	71
0.1 M phosphate		
4.7	8.3	89
9.4	16.6	88.5

DISCUSSION

Ashbya gossypii, though possessing a strong aerobic mechanism, is able also to dissimilate glucose anaerobically. Under aerobic conditions the end products of glucose oxidation are primarily carbon dioxide and water. However, small amounts of ethyl alcohol and traces of acetic acid were found when the medium was analyzed during the course of the fermentation.

Apparently the fermentation is a typical yeast alcoholic fermentation of glucose, even under aerobic conditions, but because of the strong aerobic system present the alcohol is oxidized completely to carbon dioxide as soon as the glucose is exhausted in the medium. In the 24- and 48-hour samples from the serial fermentation (table 2) the molar ratio of CO_2 and alcohol formed approached that of a typical alcoholic fermentation. Similar results were obtained

by Swanson and Clifton (1948) with *Saccharomyces cerevisiae* when growing cultures or young cell suspensions were used. No evidence of a fermentative reaction was noted with suspensions from 24-hour cultures of *A. gossypii* under aerobic conditions. Younger cultures were not tested.

Under anaerobic conditions presumably an alcoholic dissimilation occurred since 2 moles of carbon dioxide resulted from each mole of glucose metabolized. Under aerobic conditions manometric experiments showed that ethyl alcohol was oxidized by young mycelial suspensions more rapidly than was acetate or pyruvate. The oxidation of glucose was completely inhibited by low concentrations of cyanide, suggesting that the cytochrome system may be involved. Sodium fluoride and iodoacetate were also strong inhibitors of the aerobic metabolism of glucose, indicating that phosphorylated intermediates are a part of the oxidative cycle. Freezing, lyophilization, or acetone treatment of the mycelium resulted in complete loss of oxidative activity. The high endogenous respiration is probably due to fat storage in the mycelium. The R.Q. calculated on the endogenous respiration is 0.84 to 0.85, and considerable ether-soluble material is present in the mycelium.

In general, cells grown for 24 hours in a glucose medium were more active in oxidizing the substrates tested than older cells. Exceptions occurred in the cases of maltose and acetate, with which the older cells were the most active.

SUMMARY

Washed mycelial suspensions of *Ashbya gossypii* oxidize glucose, sucrose, and maltose, but not lactose. Pyruvic, acetic, and succinic acids and ethanol are all oxidized. Glucose is oxidized more or less completely to carbon dioxide and water, but in the early stages of the fermentation considerable ethyl alcohol and traces of acetic acid are formed.

Cyanide, iodoacetate, and fluoride all inhibit the aerobic metabolism of glucose.

Glucose is attacked anaerobically by mycelial suspensions, yielding 2 moles of carbon dioxide per mole of sugar consumed.

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