PRODUCTS OF ANAEROBIC GLYCEROL FERMENTATION BY STREPTOCOCCI FAECALIS

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The fermentation of oxidized or reduced substrates by homofermentative lactic acid bacteria must necessarily lead either to products other than lactic acid, or must require external hydrogen donors or acceptors. In a study of the fermentation of glycerol, a reduced substrate, by streptococci Gunsalus and Sherman (1942) noted among the enterococci two types of behavior; certain strains fermented glycerol readily with a limiting pH about 5, whereas others fermented the substrate slowly and reached a final pH of 5.5 to 6. The latter were found to require oxygen in order to utilize glycerol as an energy source. The glycerol metabolism of a strain of this type has been studied in some detail (Gunsalus and Umbreit, 1945).

In connection with the anaerobic fermentation of glycerol, Braak (1928) found with colon-aerogenes organisms that growth would cease before the glycerol was exhausted and would start again if more peptone or yeast extract was added. It seemed not unlikely that such a phenomena might also occur with lactic acid organisms.

The present paper deals with a strain of *Streptococcus faecalis* that ferments glycerol readily, with good growth, under anaerobic conditions. With this strain, yeast extract in addition to glycerol is needed for anaerobic growth, whereas with glucose as substrate yeast extract is not required. The yeast extract can be replaced with fumaric acid if a sufficient level of riboflavin is present—the fermentation products being primarily lactic and succinic acids.

METHODS

Culture. *Streptococcus faecalis*, strain 10C1, a typical enterococcus from the departmental culture collection, has been used throughout these studies. This strain, and others which ferment glycerol anaerobically, grows more abundantly in ordinary laboratory media than strains which ferment glycerol only aerobically. These strains also attack a wider range of substrates and yield a wider variety of products (Gunsalus and Campbell, 1944; Gunsalus and Niven, 1942).

Growth and media. The growth was measured turbidimetrically as described previously in papers from this laboratory (Gunsalus and Sherman, 1942). Anaerobic conditions were obtained either by vaspar seals or the chromium-sulfuric-acid method as described by Mueller and Miller (1941). The turbidity was measured at suitable intervals, and the final pH was determined at the end of the experiments, with a Beckman pH meter.

RESULTS

In order to estimate the amount of growth supported by glycerol as substrate, a comparison was made of the growth in the base medium, and in this medium with glycerol and with glucose as substrates. In these studies extra buffer was avoided in order that the influence of pH would not be further masked if slight fermentation of glycerol occurred.

Influence of Yeast Extract and Oxygen

Since glycerol is more reduced than lactic acid, and since oxygen acts as an aerobic hydrogen acceptor in glycerol fermentation, it was considered possible that anaerobically some constituent of the medium might serve as a hydrogen acceptor. To test the effect of media constituents on growth and fermentation,

TABLE 1

Effect of yeast extract and oxygen upon growth on glycerol and glucose

Streptococcus faecalis 10C1

Base Medium: 1 per cent tryptone

Incubation: 10 days, 37 C (anaerobic series in chromium-sulfuric-acid jar)

YEAST EXTRACT	AEROBIC GROWTH*			ANAEROBIC GROWTH		
	Base	Glycerol	Glucose	Base	Glycerol	Glucose
%						
0	14	21	125	12	14	125
0.2	19	52	170	19	45	160
0.5	28	70	200	30	60	170
1.0	47	115	210	44	74	190

* Turbidity 1 scale unit $\cong$ 6 μg bacterial N/10 ml.

the yeast extract level was altered as shown in table 1. One per cent tryptone supports slight growth of *Streptococcus faecalis*, strain 10C1, and glycerol improves the growth a little, whereas the addition of glucose as an energy source results in abundant growth. Not only is the anaerobic growth with glycerol poor, but aerobic growth is also slight. Therefore the tryptone must be deficient in factors necessary for the hydrogen transport to oxygen; otherwise, aerobic glycerol fermentation should occur (Gunsalus and Sherman, 1942). In the base medium yeast extract improves the growth slightly; and the further addition of glycerol provides moderate growth stimulation. The presence of oxygen affords some stimulation beyond that due to the presence of yeast extract, indicating that the quantity of hydrogen acceptor might be limiting. On the other hand, the final pH (5.0 with glycerol) may become limiting before maximum growth is attained. The growth, final pH, and titratable acidity for several variations in the medium are shown in table 2. Although very little acid was formed in the base medium, the final pH with yeast extract alone fell to 6.0. In the presence of glycerol the limiting pH was reached in all media containing 0.5 per cent, or more, yeast extract regardless of the level of tryptone. The glycerol supported about one-

third the growth, and about one-third the acid production, afforded by glucose. Whether this is due to the difference in fermentation pattern, or more likely, to the difference in growth afforded by the higher limiting pH with glycerol, cannot be determined by these data. The growth with glycerol as substrate can be about doubled by the addition of 0.5 per cent dipotassium phosphate; the limiting pH is not always reached in this case. Glycerol will support growth and acid production only when the yeast extract is added to the medium.

Fractionation of Yeast Extract and Replacement with Fumaric Acid and Riboflavin

Initial attempts to fractionate the yeast extract, as by ether extraction, resulted in two fractions neither of which was markedly active alone. However, on recombination they exhibited the full activity of yeast extract. Some fractions caused lower pH without much stimulation of growth, whereas others stimulated

TABLE 2

Anaerobic glycerol fermentation

Streptococcus faecalis 10C1

Incubation: 8 days, 37 C in anaerobic jar

TRYPTONE	YEAST EXTRACT	GROWTH*			FINAL pH			ACID PRODUCTION		
		Base	Glycerol	Glucose	Base	Glycerol	Glucose	Base	Glycerol	Glucose
%	%							*ml N/10 acid/10 ml*		
1	0	16	19	88	6.9	6.4	3.9	.1	0	3.6
1	0.5	18	56	90	6.6	5.2	4.0	0	1.1	3.9
1	1	22	58	110	6.7	5.1	4.1	0	1.6	4.6
0.5	1	18	44	74	6.6	5.0	4.1	.4	1.0	4.8
0	1	10	24	53	6.0	4.9	4.0	.4	.4	4.4

* Turbidity 1 scale unit $\cong \mu$g bacterial N.

growth without as great a depression in pH, thus suggesting the possibility that more than one substance was involved.

Therefore, several attempts were made to replace the yeast activity by known compounds. As shown in table 3, with glycerol as substrate in a tryptone medium, a small amount of yeast extract will stimulate growth only slightly but will stimulate more rapid acid production—final pH 5.0. The acid production is also stimulated by a mixture of accessory factors, or by riboflavin. In the presence of traces of yeast extract, or accessory factors, fumaric acid will greatly stimulate the growth. Although fumaric acid can replace the yeast extract, it does not necessarily follow that the action of yeast extract is due to fumaric acid.

Presumably, the fumarate acts as a hydrogen acceptor and additional riboflavin is needed for hydrogen transport. The data in table 4, with a synthetic medium, show plainly the increased riboflavin requirement. With the riboflavin and fumarate, glycerol fermentation proceeds rapidly, 12-hour growths being recorded, and appears not to require further factors beyond the requirement for growth in glucose. It should be noted, however, that the growth with glycerol as substrate still does not equal that with glucose.

TABLE 3

Factors affecting glycerol fermentation

Incubation: 2 days, 37 C Medium: 1% tryptone

ADDITIONS	GROWTH*			pH		
	Base	Glycerol	Increase	Base	Glycerol	Decrease
None	18	30	12	7.2	6.0	1.2
Yeast extract, 0.05%	20	40	20	7.2	4.9	2.3
0.1%	24	42	18	7.1	4.9	2.2
1.0%	42	76	34	6.6	4.9	1.7
Accessory factors†	20	38	18	7.2	4.9	2.3
Riboflavin, 5 μg	20	32	12	7.2	5.1	2.1
Fumarate, 0.5%	16	36	20	7.2	5.5	1.7
Fumarate Yeast extract, 0.1%	20	110	90	7.2	5.3	1.9
Fumarate Yeast extract, 0.5%	27	180	153	7.2	5.3	1.9
Fumarate Accessory factors†	15	110	95	7.2	5.3	1.9
Fumarate Riboflavin, 1 μg	20	110	90	7.2	5.4	1.8

* Turbidity 1 scale unit ≅ 6 μg bacterial N per 10 ml.

† Contains: 2.5 μg thiamine; 5 μg each of riboflavin, pyridoxine, and *para*-aminobenzoic acid; 20 μg pantothenic acid; 25 μg nicotinic acid; 1 mμg biotin; and 0.1 μg glutamine per tube.

TABLE 4

Growth and acid production in synthetic medium

Per tube: 10 ml base medium of Bellamy and Gunsalus (1945) omitting riboflavin and glucose

Incubation: 12 hours, 37 C

RIBOFLAVIN	0.5 GLUCOSE		0.5% GLYCEROL		0.5% GLYCEROL 0.5% FUMARATE	
μg/tube	Growth	pH	Growth	pH	Growth	pH
0	10	7.3	4	7.3	4	7.2
0.1	100	5.6			31	7.0
0.2	100	5.1	20	7.1	46	6.6
0.4	100	5.1	20	7.1	50	6.4
1.0	86	5.1	18	7.3	65	6.1
10.0	90	5.1	20	7.3	56	6.2

Fermentation Products from Glycerol and Glycerol-Fumarate

Fermentation balances with *Streptococci faecalis* demonstrated that approximately 95 per cent of the glucose fermented appears as lactic acid (Smith and Sherman, 1942). The fermentation pattern can, however, be altered by alkaline reaction (Gunsalus and Niven, 1942) or with oxidized substrate (Gunsalus and

Campbell, 1944). The fermentation products, with an oxidized substrate such as citric acid, are largely acetic and formic acids and carbon dioxide, with only a trace of lactic acid, thus indicating that while this organism is homofermentative on a balanced substrate, other fermentative potentialities are present. In contrast to the change in products with oxidized substrate, glycerol, a reduced substrate, yields mainly lactic acid in a tryptone yeast-extract base medium (table 5). The two extra hydrogens which arise from glycerol are largely unaccounted for.

A more marked fermentation, accompanied by increased growth, occurs in the presence of glycerol and fumarate. In this case the products are mainly lactic and succinic acids (table 5). In buffered media, especially in the presence of calcium carbonate and an excess of riboflavin, the fermentation can be further altered so that the quantity of fumarate reduced to succinate is greater than the

TABLE 5

Products of glycerol and of glycerol fumarate fermentation

Base medium: 1% tryptone, 0.2% yeast extract, 0.2% K_2HPO_4
Incubation: 3 days, 37 C Substrates added aseptically

PRODUCTS	1% GLYCEROL	1% GLYCEROL 1% FUMARATE
	mM/100 ml	*mM/100 ml*
Lactic acid	1.9	3.04
Succinic acid	0.46	3.13

Base medium: as above 1% $CaCO_3$

SUBSTRATES FERMENTED		PRODUCTS FORMED	
	mM/100 ml		*mM/100 ml*
Glycerol	7.1	Lactate	3.2
		Acetate	2.3
Fumarate	7.5	Succinate	7.5

lactic acid formed (table 5). In this case more oxidized products, acetic acid and carbon dioxide, account for the rest of the glycerol fermented. It is not surprising that under conditions in which fumarate is a good hydrogen acceptor the fermentation is altered in the direction of oxidized products, since it has previously been shown that this organism contains a very active Kreb's dismutation for the formation of acetic and lactic acids and carbon dioxide (Miller, 1942), as well as a system for the conversion of pyruvate to formic and acetic acids (Gunsalus and Campbell, 1944). This would indicate that hydrogen from triose-phosphate, as well as from the glycerol (phosphate), can be transferred to fumarate.

DISCUSSION

The anaerobic fermentation of glycerol by streptococci is dependent upon the presence of external hydrogen acceptors, the main pathway of fermentation and energy liberation proceeding by the usual lactic acid pathway. This is contrary

to the results with oxidized substrates in which a series of oxidized products are formed with energy liberation during fermentation. However, the result is similar to that found by Braak (1928) in the colon-aerogenes group. Thus it appears that these organisms are not able to carry out a more reduced type of fermentation than the lactic scheme.

The nature of the hydrogen acceptor of yeast extract that is available to this lactic organism is unknown and might bear investigation. Also, while the mechanism of the fermentation scheme with fumaric acid seems obvious, the nature of the enzymes should be determined; especially since a fumarate reductase (succinoxidase?) system in lactic acid bacteria appears not to have been previously reported.

Taxonomic considerations could call for a review of the relationship of the aerobic and anaerobic glycerol fermentation types of enterococci to *Streptococcus foecium* and *Streptococcus glycerinaceous*, respectively, of Orla-Jensen (1919).

SUMMARY

Glycerol fermentation by streptococci has been found to occur only in the presence of external hydrogen acceptors, the main reaction being:

$$\text{glycerol} \rightarrow \text{lactic acid} + 2H$$

Some strains, as described previously, can use only oxygen as a hydrogen acceptor, the other product being H_2O_2.

Other strains, as reported in this study, can use an unidentified substrate in yeast extract as hydrogenacceptor. This can be replaced by fumaric acid, in which case the main reaction becomes:

$$\text{glycerol} + \text{fumaric acid} \rightarrow \text{lactic acid} + \text{succinic acid}$$

This reaction requires a higher riboflavin level than is necessary for glucose fermentation, very probably for hydrogen transport to fumaric acid. With an excess of fumarate, oxidized products are formed.

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