OXIDATIVE METABOLISM IN *PEDIOCOCCUS PENTOSACEUS*

I. Role of Oxygen and Catalase[1, 2]

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ABSTRACT

Dobrogosz, Walter J. (Pennsylvania State University, University Park) and Robert W. Stone. Oxidative metabolism in *Pediococcus pentosaceus*. I. Role of oxygen and catalase. J. Bacteriol. **84**:716-723. 1962.—Studies were conducted on the physiological behavior of several strains of *Pediococcus pentosaceus* isolated from alfalfa silages. Although these organisms are regarded as homofermentative lactic acid bacteria which metabolize carbohydrates via the classic reactions of glycolysis, this investigation showed that they were capable of developing other physiologically important reactions related to carbohydrate metabolism. Growth on glycerol, for example, was shown to depend on the development of aerobic reactions, and was directly related to the catalase content of the various strains tested. These organisms were shown to be devoid of a cytochrome system, thus implicating an active flavoprotein system in oxidative reactions. A study of the end products of aerobic glycerol metabolism suggested that glycerol was oxidized to the pyruvate level, with subsequent reactions involving pyruvate leading to the accumulation of lactate, acetate, acetoin, and CO_2 in a molar ratio of approximately 1:1:1:3.

There is little information available on the physiology of members of the genus *Pediococcus*. They are known to be gram-positive, tetrad-forming cocci which are considered to be microaerophilic and members of the family *Lactobacillaceae* (Pederson, 1949; Jensen and Seeley, 1954). When grown in rich carbohydrate media, they are known to exhibit a homolactic fermentation pattern (Pederson, 1949; Christensen, Albury, and Pederson, 1958; Nakagawa and Kitahara, 1959), and under certain conditions have been shown to form diacetyl (Christensen and Pederson, 1958). In addition, some strains are known to possess catalase activity (Felton, Evans, and Niven, 1953; Delwiche, 1961) and to ferment pentoses to equimolar amounts of lactate and acetate (Fukui et al., 1957).

In contrast to the relatively sparse information on their physiological behavior, these organisms have been implicated as important ecological agents in a wide variety of fermentation processes, such as used in the brewing industry (Shimwell and Kirkpatrick, 1939; Iizuka and Yamasato, 1959) and as starter cultures in sausage fermentations (Deibel and Niven, 1957), and have recently been shown to be of possible importance in silage fermentations (Dobrogosz and Stone, 1958).

The present investigation was undertaken to study the metabolic reactions of these bacteria with particular emphasis on the roles of catalase and oxygen in their physiological behavior.

MATERIALS AND METHODS

All cultures used in this study were isolated by the authors (Dobrogosz and Stone, 1958) from fermenting alfalfa silages. The cultures were identified as members of the genus *Pediococcus*, by use of the morphological and physiological criteria of Breed, Murray, and Smith (1957) and Nakagawa and Kitahara (1959). According to the former classification, all cultures used in this study were strains of the species *Pediococcus cerevisiae*; whereas, according to Nakagawa and Kitahara (1959), the species designation, *P. pentosaceus*, is applicable to these cultures. Since the more recent classification of

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Nakagawa and Kitahara appears to be based on a more detailed study of this genus, the cultures used in this study will be referred to as *P. pentosaceus*.

Stock cultures were maintained by monthly transfers in Tomato Juice Agar (Difco). The growth medium used in these studies had the following basal composition (in g/liter): N-Z-Case (Sheffield Chemical, Norwich, N.Y.), 10.0; Yeast Extract (Difco), 10.0; K_2HPO_4, 1.0; NaCl, 4.0; $MgSO_4 \cdot 7H_2O$, 0.16; $FeSO_4 \cdot 7H_2O$, 0.008; $MnCl_2 \cdot 4H_2O$, 0.003. The medium was adjusted to pH 7.0, and was sterilized separately from the added carbohydrates. The basal medium shall be referred to as NYE basal.

Approximately 0.25 ml of the stock culture (grown for 16 hr in NYE basal containing 0.10% glucose) was inoculated into 100 ml of the growth medium and allowed to incubate at 37 C for 16 to 24 hr on a reciprocating shaker. Growth was measured turbidimetrically, using an Evelyn colorimeter and a 660 mμ filter. Under the experimental conditions used, the optical density of the freshly inoculated medium was not significantly different from that of the uninoculated medium. Aerobiosis during growth was maintained by shaking in cotton-plugged 500-ml Erlenmeyer flasks. Anaerobiosis was attained by flushing the inoculated flasks with sterile nitrogen and sealing them with tight-fitting rubber stoppers. That this procedure provided anaerobic conditions throughout the growth period was indicated by the maintenance of the reduced state of methylene blue solutions treated in this manner and by the inability of cultures to grow on glycerol under these conditions. Growth on this substrate requires an aerobic atmosphere.

Catalase activities were measured manometrically with the Warburg apparatus, using washed, whole cells suspended in 0.067 M phosphate buffer (pH 6.9) to a standard optical density of 0.230 at 660 mμ. The reaction system consisted of standard cell suspension, 0.20 ml; H_2O_2, 440 μmoles; phosphate buffer (pH 6.9), 134 μmoles; volume to 3.0 ml. The temperature was 30 C. The rate of oxygen evolution was followed and the relative catalase activities were expressed as μliters of O_2 evolved per hr per 0.20 ml of standard cell suspension. Corrections were made for endogenous activity and spontaneous decomposition of the H_2O_2.

All procedures used in the carbon balance studies were those of Neish (1952). Glucose was determined by the method of Morris (1948), and glycerol by the method of Lambert and Neish (1950). Diacetyl was determined by the method of White, Krampitz, and Werkman (1946), and acetoin by the methods of Westerfield (1945) and Eggleston, Elsden, and Gough (1943). Lactic, acetic, and formic acids were determined by the column-chromatographic method of Wiseman and Irvin (1957).

A modification of the procedure of Lenhoff and Kaplan (1956) was used for the determination of cytochrome components in crude extracts. The cells were washed twice in 0.067 M phosphate buffer (pH 7.2) and frozen at -17 C. The frozen pellets were ground with an equivalent weight of alumina and diluted with 6 ml of additional phosphate buffer per g of wet cells. The preparation was centrifuged at 2,000 $\times$ *g* for 15 min and then at 25,000 $\times$ *g* for 40 min; 3 ml of the supernatant fraction thus prepared was reduced with a few crystals of sodium hydrosulfite, and optical density was determined at various wavelengths with a Beckman model DU spectrophotometer. An automatic-recording Warren Spectracord (Warren Electronics, Inc.) was used in recording the absorption peaks.

RESULTS

Gunsalus and Sherman (1943) and Gunsalus and Umbreit (1945) reported that certain lactic acid bacteria were capable of utilizing glycerol only under aerobic conditions. A similar requirement for oxygen in pediococcal metabolism was thus suggested. In this connection, therefore, glucose and glycerol utilization were studied under both aerobic and anaerobic conditions; six catalase-positive and six catalase-negative strains were used, and utilization of glycerol was measured by the acidity produced in terms of pH. The results (Table 1) demonstrated that glycerol utilization under these conditions was an obligate aerobic process; in a similar experiment, glucose was utilized to an equivalent extent under either aerobic or anaerobic conditions. With the possible exception of strains 4 and 4M, these results also indicated that catalase content was possibly correlated with the extent of glycerol utilization, since the positive strains appeared to be more active. This was particularly evident in medium B, which was the standard NYE basal medium used throughout the remainder of these studies.

TABLE 1. *Effect of aerobic and anaerobic growth on glycerol utilization by catalase-positive and catalase-negative Pediococcus strains*

Strain no.	Catalase[b]	ΔpH[a]		
		Medium A[c]		Medium B[d]
		Aerobic	Anaerobic	Aerobic
65	+	−1.95	+0.03	−2.00
Az-5-6	+	−1.67	+0.04	−2.10
Az-25-5	+	−1.52	+0.11	−2.12
TJ-10-1	+	−1.80	+0.04	−2.02
TJ-9-1	+	−1.52	+0.08	−2.06
TJ-14-9	+	−1.71	+0.10	−2.16
64	0	−0.53	+0.02	−0.27
4	0	−1.26	+0.12	−1.67
4M	0	−1.62	+0.06	−1.37
67	0	−0.45	+0.01	−0.24
Az-37-4	0	−1.01	+0.03	−0.40
Az-5-1	0	−1.00	+0.04	−0.34

[a] That is, pH units below (−) or above (+) values for the basal medium alone after 72 hr of incubation.

[b] Determined by a qualitative assay based on flooding growth plates with 3% H_2O_2.

[c] NYE basal medium containing 0.75% glycerol, but without addition of the mineral salts.

[d] NYE basal medium containing 0.75% glycerol.

To define more clearly the relation of catalase to glycerol utilization, three positive and three negative strains were grown aerobically in various concentrations of glycerol for 90 hr. The pH values were measured and recorded (Fig. 1). These results showed that the catalase-positive strains were capable of metabolizing larger amounts of glycerol than were the negative strains.

These data (Fig. 1) suggested that a quantitative relationship might exist between the catalase content of the various strains and their respective abilities to grow on glycerol. This possibility was tested by measuring the optical densities and pH values after the cultures were grown aerobically for 90 hr in 1.5% glycerol medium. These values were then compared with the relative catalase levels in each strain after 19 hr of growth under the same original conditions. With the possible exception of strain 4, these results (Table 2) indicated that the ability of these strains to grow on glycerol was in direct proportion to their relative catalase activities.

That the relationship between catalase content and glycerol utilization was not merely fortuitous was shown by the experiments recorded in Fig. 2. In these experiments, exogenous catalase (Nutritional Biochemicals Corp., Cleveland, Ohio, technical grade) was added (0.25 ml per 100 ml of culture medium) to the six strains at the time of their inoculation into media containing 1.5% glycerol. Heat-inactivated catalase (heated for 15 min at 100 C) was added to an identical set of cultures as a control series. Growth was then followed during a 140-hr incubation period. It can be seen from these data that, whereas

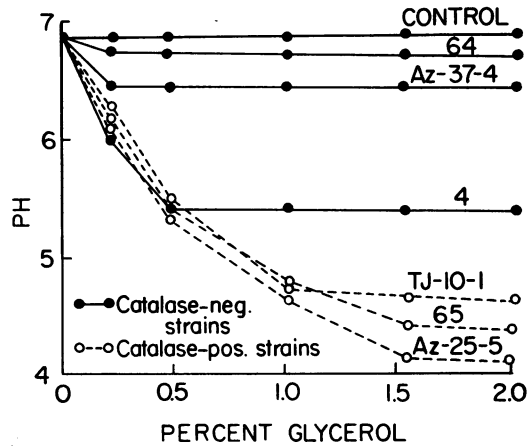

FIG. 1. *Glycerol utilization by catalase-positive and catalase-negative strains of pediococci.*

TABLE 2. *Ability of Pediococcus strains to utilize glycerol correlated with their respective catalase contents*[a]

Strain no.	Optical density (660 mμ)	pH	Catalase activity[b]
64	0.09	6.8	0
Az-37-4	0.17	6.5	0
4	0.43	5.4	0
TJ-10-1	0.58	4.6	390
65	0.77	4.4	670
Az-25-5	0.79	4.1	1,500

[a] All cultures were grown in NYE basal medium containing 1.5% glycerol and under aerobic conditions. The cultures which were to be assayed for catalase activities were grown for 19 hr. For the optical density and pH measurements, the cultures were grown for 90 hr.

[b] Expressed as μliters of O_2 evolved per hr per 0.20 ml of standard cell suspension.

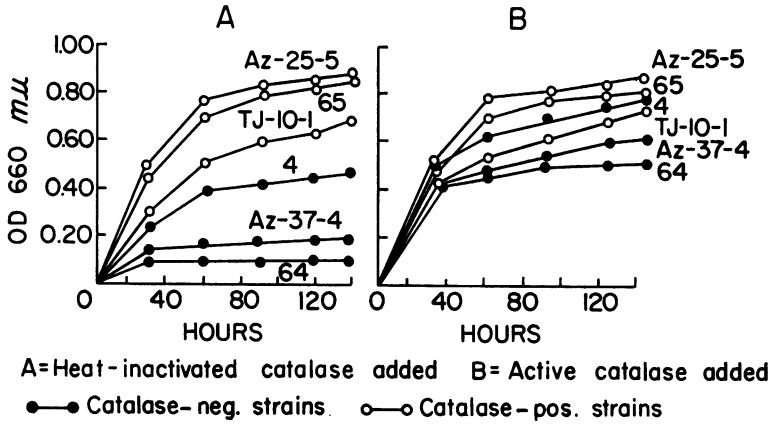

FIG. 2. *Effect of exogenous catalase on growth of six Pediococcus strains.*

heat-inactivated catalase was without effect, active catalase greatly stimulated growth of those strains which were deficient in, or apparently devoid of, this enzyme (Table 2). A physiologically significant role for catalase (and an active flavoprotein oxidative system) during glycerol metabolism by these organisms was thus indicated.

The obligate aerobic growth on glycerol, the possession of catalase by some strains, and an indication of a positive Nadi reaction for cytochrome *c* in these bacteria (Jensen and Seeley, 1954) suggested the possibility that a cytochrome system may be present in these organisms.

To test this possibility, a spectrophotometric analysis of reduced cytochrome pigments was undertaken. The most aerobically active strain (Az-25-5) was used for this purpose. Inasmuch as the streptococci are devoid of a cytochrome system (Farrell, 1935), *Streptococcus faecalis* B33A was chosen as a negative control culture. *Pseudomonas fluorescens* and *Bacillus subtilis* were chosen as positive control cultures. All cultures were grown aerobically in 1.5% glycerol medium for 48 hr. Cell-free extracts were prepared and examined as previously described.

The results of this study are presented in Fig. 3. Although absorption peaks were evident at approximately 550, 520, and 415 mμ for the positive controls, strain Az-25-5 and *S. faecalis* manifested no significant absorption between 400 and 700 mμ. Therefore, no evidence was obtained for a cytochrome system in this strain of *Pediococcus*. Although other strains were not tested, it is quite likely that the pediococci, as all other genera of the *Lactobacillaceae* which have been investigated in this connection, are devoid of a cytochrome-type electron-transport system.

During the course of these studies, it was repeatedly observed that a strong butterlike odor, characteristic of diacetyl, was associated with growth in glycerol but not with growth on glucose. This observation suggested that, in addition to an oxygen requirement, glycerol metabolism may also be distinguished from glucose metabolism in these organisms by the end products formed. Although it had been determined that pediococci are homofermentative lactic acid producers when grown in high concentrations of glucose, fructose, or sucrose (Christensen and Pederson, 1958), the products of aerobic glycerol metabolism have not been previously reported.

Carbon balances were determined after cells of strain Az-25-5 were grown for 48 hr aerobically on glycerol, and aerobically and anaerobically on glucose (Table 3).

These data showed that aerobic or anaerobic growth on glucose was accompanied by the production of 79 to 84 mmole per cent of lactic acid. It appears that, under aerobic or anaerobic conditions, hexose degradation is typically of the homolactic type. During glycerol utilization, however, lactic acid accounted for only 13 mmole per cent of the products determined. Utilization of glycerol resulted in lactic acid, acetic acid, acetoin, and CO_2 being produced in a ratio of approximately 1:1:1:3, respectively. A low level of diacetyl production was also noted. The over-all equation for glycerol metabolism suggested that pyruvate formed by the oxidation of glycerol can

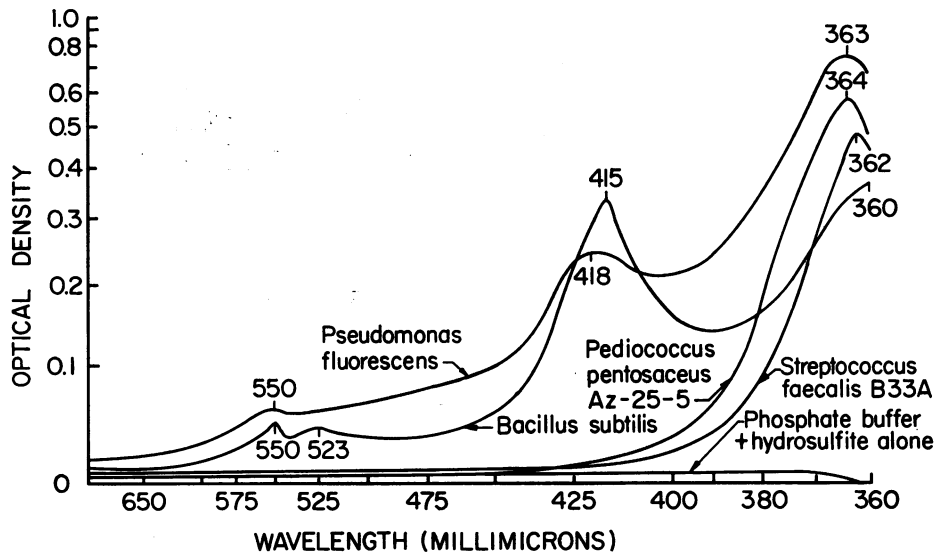

FIG. 3. *Analysis of cytochrome pigments in Pediococcus pentosaceus Az-25-5.*

TABLE 3. *Products of glucose and glycerol degradation by Pediococcus pentosaceus Az-25-5*[a]

Product	Glycerol (aerobic)	Glucose (aerobic)	Glucose (anaerobic)
Lactic acid	20.7	187	170
Acetic acid	29.9	18.6	12.9
Carbon dioxide	75.0	20.1	7.8
Formic acid	3.9	9.1	10.5
Acetoin	27.4	3.4	0.3
Diacetyl	0.9	0	0
Per cent carbon recovered	104	106	93
Millimole per cent of products other than lactic acid	87	21	16

[a] Growth was conducted in the NYE basal medium containing either 0.163 M glucose or glycerol for 48 hr under conditions of sparging with O_2 (aerobic) or N_2 (anaerobic). Results are expressed as mmoles of product formed per 100 mmoles of substrate utilized.

be further metabolized via two well-established routes in addition to the reduction to lactate: oxidation to acetate and CO_2, and the formation of acetoin and CO_2 via the decarboxylation of α-acetolacetate, which is formed by the condensation of two moles of pyruvate with the liberation of one mole of CO_2.

DISCUSSION

The pediococci are regarded as homofermentative lactic acid bacteria capable of equivalent growth under either aerobic or anaerobic conditions. The carbon balance studies with glucose as substrate reported in this work are in agreement with this view. Additional data were obtained, however, which clearly indicate that, although these organisms are devoid of a cytochrome system, they are nevertheless capable of manifesting a physiologically significant oxidative system during growth on glycerol. The observed requirement for catalase during aerobic glycerol metabolism further suggests that these organisms utilize a flavoprotein enzyme system for the transport of electrons to oxygen, resulting in hydrogen peroxide production. In this connection, Dolin (1961) recently reviewed the significance of oxidative metabolism in organisms such as the lactic acid bacteria, which do not contain cytochromes.

Although the intermediate steps in glycerol dissimilation by the pediococci were not directly investigated in these studies, an examination of the data which have been presented, considered in light of the information available on the glycerol metabolism in *Streptococcus faecalis* 10C1 (Jacobs and Van Demark, 1960), suggested a tentative pathway for glycerol metabolism in *P. pentosaceus* as illustrated in Fig. 4. The close

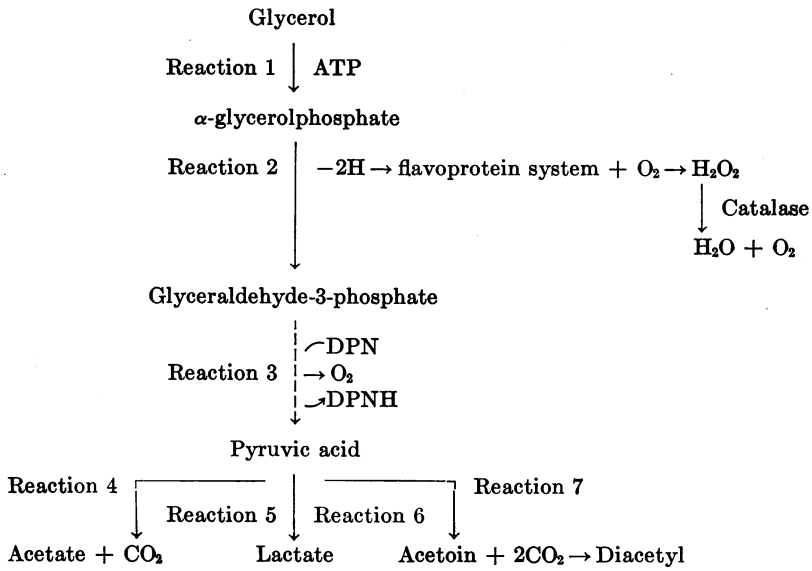

FIG. 4. *Tentative pathway for glycerol metabolism in Pediococcus pentosaceus.*

physiological relationship of *S. faecalis* strains to *Pediococcus* strain Az-25-5 has been noted throughout the course of this study. It is likely, therefore, that many of the reactions involved in the metabolism of glycerol by *S. faecalis* are similar to those involved in the metabolism of this substrate by the pediococci.

Reaction 1 (Fig. 4), in which glycerol reacts with adenosine triphosphate, was demonstrated with *S. faecalis* 24 by Gunsalus and Umbreit (1945). A similar glycerol kinase was reported in cell-free extracts of aerobically grown *S. faecalis* 10C1 by Jacobs and Van Demark (1960). Reaction 2 is more complicated. Gunsalus and Umbreit (1945) suggested that the dehydrogenation of α-glycerolphosphate (α-GP) was not linked to diphosphopyridine nucleotide (DPN), since pyruvate was not reduced during dissimilation of glycerol or α-GP under anaerobic conditions. It was suggested that this α-GP-oxidizing system was linked directly to oxygen via a flavoprotein-mediated system with H_2O_2 as one of the products. The observation by Jacobs and Van Demark (1960) that cell-free extracts of aerobically grown *S. faecalis* 10C1 possessed a non-DPN-linked α-GP oxidase appears to support this interpretation. This enzyme was shown to oxidize α-GP, with atmospheric oxygen as a hydrogen acceptor, forming H_2O_2 as an end product. The importance of catalase for the aerobic utilization of glycerol by *P. pentosaceus* is indicative that a similar H_2O_2-generating reaction may occur in the pediococci. The necessity for oxygen and catalase in their metabolism of glycerol can be understood in light of these reactions.

Reaction 3 refers to the triose-phosphate dehydrogenation and subsequent reactions of the Embden-Meyerhof glycolytic pathway, culminating in pyruvate formation. Jacobs and Van Demark (1960) have shown that extracts of either aerobically or anaerobically grown *S. faecalis* 10C1 contained a DPN-linked triose-phosphate dehydrogenase. Although pyruvate is probably the main acceptor for the DPNH generated during the triose-phosphate dehydrogenation, Dolin (1961) demonstrated a series of flavin enzymes which can mediate the oxidation of DPNH with oxidants such as oxygen, cytochrome *c*, and ferricyanide.

Further metabolism of pyruvate is considered in reactions 4 through 6. The results presented in Table 3 show that *P. pentosaceus* grown aerobically on glycerol produced lactic acid, acetic acid, acetoin, and CO_2 in a molar ratio of 1:1:1:3, respectively. These products can be accounted for by assuming the conversion of glycerol to pyruvate by the reactions discussed above and the subsequent reactions of pyruvate as follows:

reaction 4: pyruvate + $\frac{1}{2}O_2$ → acetate + CO_2 + H_2O
reaction 5: pyruvate + 2H → lactate
reaction 6: 2 pyruvate → α-acetolactate + CO_2
α-acetolactate → acetoin + CO_2
reaction 7: acetoin − 2H → diacetyl

The theoretical sum of these reactions, excluding the small amount of diacetyl formed, yields the following equation:

4 glycerol → 1 lactate + 1 acetate + 1 acetoin + $3CO_2$

The carbon balance for glycerol dissimilation by *P. pentosaceus* resulted in the following equation:

4 glycerol → 0.83 lactate + 1.2 acetate + 1.1 acetoin + $3CO_2$

Since all these reactions (1 to 7) have been previously demonstrated in *S. faecalis* 10C1 (Dolin and Gunsalus, 1951; O'Kane, 1950; Gunsalus, Horecker, and Wood, 1955), it is likely that they are also involved in the dissimilation of pyruvate by the pediococci.

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