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CATALASE ACTIVITY OF TWO *STREPTOCOCCUS FAECALIS* STRAINS AND ITS ENHANCEMENT BY AEROBIOSIS AND ADDED CATIONS[1]

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ABSTRACT

JONES, DOROTHY (American Meat Institute Foundation, Chicago, Ill.), R. H. DEIBEL, AND C. F. NIVEN, JR. Catalase activity of two *Streptococcus faecalis* strains and its enhancement by aerobiosis and added cations. J. Bacteriol. **88**:602-610. 1964.—The nature of catalase activity noted in two unusual *Streptococcus faecalis* strains was determined. Enzyme activity was lost slowly when cultures were maintained by daily transfer in test tubes of broth media. Loss of activity could be prevented by aerobic culture. Supplementation of the growth medium with ferric, manganese, and zinc ions, as well as aerobiosis, enhanced catalase activity. However, addition of these cations to cell suspensions or to cell-free extracts did not increase catalase activity. Although oxygen was observed to be one of the reaction end products, the catalase activity was not inhibited by cyanide or azide, and the iron-porphyrin coenzyme of classical catalase was not detected. The enzyme was purified 185-fold by precipitation with ammonium sulfate, followed by chromotography on a diethylaminoethyl cellulose column.

Catalase is considered to be absent characteristically from the family Lactobacillaceae. However, studies with the genus *Pediococcus* (Felton, Evans, and Niven, 1953) revealed that this group appeared to differ from other lactic acid bacteria in that catalase activity was demonstrable in some strains. Subsequently, other investigators observed catalase-like activity among strains representing several species of the Lactobacillaceae (Vankova, 1954; Wheater, 1955; Dacre and Sharp, 1956; Deibel and Niven, 1960; Whittenbury, 1960; Langston, Gutierrez, and Bouma, 1960). The incidence of this characteristic is not common, and activity can be detected only under specific conditions of culture. Usually, the medium of choice is one containing a low concentration of glucose (Felton et al., 1953; Gutekunst, Delwiche, and Seeley, 1957). Whittenbury (1960) reported increased catalase activity in some species when heated blood was incorporated in the medium. In these instances, the catalase activity of the cultures was not influenced appreciably by the quantity of glucose added to the growth medium.

Iron-porphyrin compounds have not been detected in the Lactobacillaceae (Deibel and Evans, 1960) and the prosthetic group of the catalase-like enzyme has not been determined. Delwiche (1959, 1961), working with the catalase activity of *P. cerevisiae*, was unable to demonstrate any inhibition with azide or cyanide.

The present study was undertaken to determine the optimal conditions for the production of the catalase-like activity in two strains of *Streptococcus faecalis* which possessed this unusual enzyme. In addition, some characteristics of a partially purified preparation of the enzyme are reported.

MATERIALS AND METHODS

Source of strains. Two catalase-positive strains of *S. faecalis* (T91 and P318) were obtained from C. W. Langston, U.S. Department of Agriculture, who had isolated them from silage. The organisms were characterized physiologically and serologically, and the results regarding their taxonomy confirmed those of Langston et al. (1960).

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Other microorganisms employed in this study were obtained from the stock culture collection of the American Meat Institute Foundation.

Media. Stock cultures were maintained in a yeast extract-Tryptone-glucose broth supplemented with citrate, inorganic salts, and Tween 80, commonly referred to as APT broth (Difco). After preliminary experiments, strains were maintained by daily transfer on agar slopes of the low glucose medium described by Felton et al. (1953). This medium was employed also as the experimental growth medium in this study.

Conditions of incubation. Aerobic conditions were achieved by incubating cultures on a reciprocal shaker. Stationary conditions refer to incubation of cultures in liquid media without shaking. Anaerobic cultures were incubated in an atmosphere of 95% nitrogen-5% CO_2 in a 6-liter desiccator after flushing three times with this gas mixture.

Measurement of growth. Matched tubes (18 mm in diameter) were used, and growth was estimated by optical density measurements in a Bausch & Lomb Spectronic-20 colorimeter at 600 mμ.

Preparation of cell-free extracts. Aerobically grown, 24-hr cultures were centrifuged at 4 C. The packed organisms were washed once in 0.033 M phosphate buffer (pH 7.0), resuspended in 0.025 volume of the same buffer, and disintegrated in a Raytheon sonic oscillator (10 kc) for 30 min in portions of 100 ml. The disintegrated cells were pooled and centrifuged at 3,000 × *g* for 30 min at 4 C. The supernatant fluid constituted the cell-free extract. Protein determinations in these extracts were made by the method of Stadtman, Novelli, and Lipman (1951). In purification procedures, enzyme activity was recorded as units per milligram of protein, as defined in Table 4.

Enzyme assay. Oxygen evolution was measured by conventional manometric techniques (Umbreit, Burris, and Stauffer, 1957). Whole cells or cell extracts were placed in the main compartment of the reaction vessel, and the volume was adjusted to 2.6 ml with 0.033 M phosphate buffer (pH 7.0). Hydrogen peroxide (26 μmoles in 0.2 ml of 0.033 M phosphate buffer) was added to the side arm, and the center well contained 20% potassium hydroxide (0.2 ml).

For comparative purposes, beef liver catalase (Armour) was employed. In these experiments, approximately 13 μmoles of oxygen (the theoretical quantity from 26 μmoles of hydrogen peroxide) were evolved in a 30-min period.

TABLE 1. *Comparison of catalase activity of Streptococcus faecalis T91 and Pediococcus cerevisiae E66*

Organism	Glucose concn*	Final pH	pH of suspending medium	Enzyme activity†
	%			
P. cerevisiae E66.....	0.5	4.8	4.8	1.0
			7.0	2.8
	0.05	6.6	6.6	4.2
			7.0	5.5
S. faecalis T91.....	0.5	4.4	4.4	0
			7.0	0
	0.05	6.4	6.4	1.0

* Cells were grown in basal medium (stationary culture for 24 hr at 37 C) containing the indicated glucose concentrations.

† Manometric assay with the use of cell suspensions. Figures refer to micromoles of O_2 evolved from 26 μmoles of H_2O_2 in 30 min.

RESULTS

Effect of glucose and pH value. S. faecalis T91 and P318 were streaked on agar plates containing 0.05 and 0.5% glucose. After incubating for 24 hr, the plates were flooded with 3% hydrogen peroxide and catalase activity was estimated visually. Activity was very evident in both strains when grown on the low glucose medium, but both strains demonstrated radically diminished activity on the 0.5% glucose medium.

In an attempt to quantitate these differences, *S. faecalis* T91 was cultured in broth media (stationary cultures) containing 0.05 and 0.5% glucose. For comparative purposes, *P. cerevisiae* E66 was included. After growth for 24 hr at 37 C, samples of the cultures were removed and adjusted to equal optical density readings (i.e., 0.7) with uninoculated media of the same pH. The cells in the remaining cultures were centrifuged, washed in 0.033 M phosphate buffer (pH 7.0), and adjusted to the same optical density (0.7) in the phosphate buffer. Catalase activity of all suspensions was assayed manometrically.

The results were similar to those obtained by Delwiche (1959) with the *P. cerevisiae* strain (Table 1). Catalase activity of *P. cerevisiae* was depressed when grown in 0.5% glucose medium and tested in cell suspension at pH 4.8 but higher

TABLE 2. *Catalase activity of Streptococcus faecalis T91 grown under different cultural conditions*

Incubation	Glucose concn*	Final pH	Cell suspension medium†	Enzyme activity‡
	%			
Aerobic.....	0.5	5.3	Growth medium	2.2
			Buffer	2.1
Stationary.....	0.5	4.4	Growth medium	0
			Buffer	0
Aerobic.....	0.05	6.8	Growth medium	3.7
			Buffer	4.1
Stationary.....	0.05	6.6	Growth medium	1.3
			Buffer	1.3

* Cells were grown in basal medium with indicated glucose concentration for 24 hr at 37 C.

† The optical density of all cell suspensions was adjusted to approximately 0.7 with uninoculated medium (pH value equivalent to final pH of medium) or 0.033 M phosphate buffer (pH 7.0).

‡ Manometric assay. Figures refer to micromoles of O_2 evolved from 26 μmoles of H_2O_2 in 30 min.

enzyme activity was evident when the same cells were resuspended in 0.033 M phosphate buffer at pH 7.0. Catalase activity of *S. faecalis* T91 cell suspensions was also depressed when grown in a medium containing 0.5% glucose. Furthermore, activity was not regained when the cells were resuspended in 0.033 M phosphate buffer at pH 7.0. In addition, under the conditions of growth employed in this experiment, the catalase activity of *S. faecalis* appeared to be diminished when compared to cultures grown on semisolid media. Thus, from these results it appeared that additional factors other than pH value and glucose concentration were involved in the catalase activity of *S. faecalis* T91.

Loss of activity. In the early phases of this study, the *Streptococcus* strains were maintained by daily transfer in APT broth. As the cultures were carried, the catalase activity became weaker and finally disappeared completely. Alteration of the medium constituents, including the use of tomato juice (Langston and Bouma, 1960), failed to restore the activity, even though the organisms grew promptly and maximally in these media. New frozen stocks were thawed and carried in various media, and the identical loss of activity was noted. At this point it was observed that stocks maintained on agar slants consistently possessed higher activity than did broth cultures, and activity was not lost upon serial culture.

Although a loss of activity was prevented by aerobic culture, all attempts failed to restore catalase activity among those cultures which lost this property. Such cultures were carried for 3 months by daily subculture on agar slopes; however, no catalase activity was detected at any time.

An attempt was made to induce catalase activity among other enterococci. Three stock strains of *S. faecalis* and four of *S. faecium*, known to be catalase-negative, were subcultured daily on agar slopes for 6 months. At intervals these organisms were streaked on plates of the basal medium as well as agar media of varying composition. Cell suspensions were also employed and activity was measured manometrically. In no instance was unequivocal catalase activity demonstrated among these strains.

Effect of oxygen tension. The catalase activity of *S. faecalis* T91 grown in aerobic and stationary cultures containing 0.05 and 0.5% glucose was compared. The cells were assayed both in the growth medium and in 0.033 M phosphate buffer (pH 7.0) at equivalent optical densities (Table 2). Aerobiosis, coupled with a low glucose concentration, significantly enhanced activity. In a medium containing 0.5% glucose, the pH fell to 4.4 in stationary cultures, and there was little or no catalase activity. However, in aerobically grown cultures, the pH did not fall below 5, and there was demonstrable catalase activity. In a medium containing 0.05% glucose, there was little difference in the final pH of the aerobically and anaerobically grown cultures, although catalase activity of the aerobically grown culture was approximately three times that of the stationary culture. These results, in addition to the observed loss of activity during serial subculture in broth media as discussed previously, indicate that a high oxygen tension during growth enhances synthesis of the enzyme.

Enhancement of activity by inorganic ions. In the early phases of this study, stimulation of catalase activity was noted when salts of manganese were added to semisolid agar media. The basal medium was altered in that 0.5% sodium citrate was added to maintain the inorganic salts in solution. Plate cultures were developed by adding hydrogen peroxide (3%), and the activity

of the cultures grown on the basal medium was compared visually with cultures grown in media supplemented with cations. Among the cations tested, only manganese, ferric iron, and zinc resulted in enhanced catalase activity. Other cations tested were inactive. In addition to its increased catalase activity, surface growth on the media supplemented with cations was colored (Jones, Deibel, and Niven, 1963). Other *S. faecalis* strains also demonstrated coloration, but only strains T91 and P318 exhibited catalase activity under these conditions.

An attempt was made to quantitate the increased enzyme activity by growing cultures of *S. faecalis* T91 aerobically in the basal medium (0.5% sodium citrate added) with no added cations, and in the same medium containing approximately 1 mmole per liter of the individual cations. After incubation for 24 hr, the cultures were centrifuged (3,000 × *g* for 30 min at 4 C), and the cells were washed once and resuspended in 0.033 M phosphate buffer (pH 7.0). After adjusting the cell crops to equivalent optical densities, samples were removed and heated (10 min at 99 C) for control purposes. All preparations were assayed manometrically for catalase activity.

Cells grown with the added cations evidenced the greatest activity, and the enhancement of activity did not differ significantly among the three cations tested. Heated cell suspensions were inactive.

In another series of experiments, *S. faecalis* T91 was grown aerobically in the basal medium without added cations. In these experiments, no increased activity was noted when 1, 5, and 10 μmoles of Fe^{+3}, Mn^{+2}, or Zn^{+2} were added to the cell suspensions immediately prior to assay. Moreover, incubation of cell preparations for 1 and 2 hr with the cations prior to assay failed to increase catalase activity. Consequently, it was concluded that the cations must be present during active growth of the organism for the production of maximal enzyme activity.

Because of the coloration of the cells imparted by growth in the presence of added inorganic ions, some doubt could be expressed regarding the accuracy of optical density and dry weight determinations. To obtain more reliable data, the experiment previously described, in which the growth medium was supplemented with approximately 1 mmole per liter of the respective cations, was repeated. After 24 hr at 37 C, the cells were harvested, washed, and sonically disrupted (30 min in phosphate buffer). After centrifugation (3,000 × *g* for 15 min at 4 C), the protein content of the supernatant fluids was determined by the method of Stadtman, Novelli, and Lipmann (1951). Significant differences in the activity of the various cell-free preparations were noted (Table 3).

TABLE 3. *Catalase activity of cell-free extracts (Streptococcus faecalis T91) derived from cultures grown with various cationic supplements*

Cation added to growth medium	Protein in extract	Enzyme activity*	Specific activity†
	mg/ml		
Mn^{++}	2.7	67.5	25.0
Fe^{+++}	3.8	35.0	9.2
Zn^{++}	3.8	9.5	2.5
None	3.8	2.0	0.5

* Manometric assay. Figures refer to micromoles of O_2 evolved per milliliter of cell-free preparation during the first 5 min of assay.

† Specific activity defined as enzyme activity per milligram of protein per milliliter of preparation.

Because relatively high concentrations of the inorganic ions were employed in the growth media, attempts were made to determine the concentration required for maximal catalase activity. In these experiments ferric iron was chosen, although greater stimulation was observed with manganese salts. This choice precluded the possible involvement of manganese salts (i.e., MnO_2) which might decompose hydrogen peroxide nonenzymatically. However, it must be emphasized that the concentrations of manganese sulfate employed in manometric studies with cell suspensions or cell-free extracts never evidenced activity in this respect.

Cultures containing 0 to 4.3 mmoles of added Fe^{+3} per liter were incubated aerobically for 24 hr at 37 C. Cell-free extracts were prepared, and catalase activity was estimated as described previously. Maximal activity was observed when approximately 1.44 mmoles per liter were added. In subsequent studies, this concentration of iron was added to the test media. No attempts were made to estimate the quantity of cations that were present in the basal medium.

Effect of hemin. Whittenbury (1960) observed increased catalase activity for various lactic acid

bacteria when heated blood was added to the growth medium. Subsequently, Delwiche and Johnson (1962) noted enhanced activity when *P. cerevisiae* E66 was grown in the presence of hemin. When *S. faecalis* T91 and P318 were grown on Whittenbury's medium and the activity was estimated visually, an apparent slight enhancement was noted; however, the uninoculated plates also exhibited some activity.

The effect of hemin and iron on the catalase activity of *S. faecalis* T91 was compared by growing the organism in the basal medium and in the same medium supplemented with 2 mg of hemin (Armour) per liter of medium or with 1.44 mmoles per liter of ferric iron. Cell-free extracts were prepared and catalase activity was estimated as described previously. Hemin stimulated activity; however, the stimulation was approximately one-third that obtained with iron.

The possibility existed that the hemin stimulated activity by virtue of its iron content. To test this hypothesis, 150 mg of hemin were added to 1 liter of growth medium. This concentration gave an equivalent iron concentration of 50 mg of ferric sulfate. The activity of cell-free extracts prepared from the organism grown under these conditions revealed an enhanced activity over that observed with ferric iron supplementation. However, it was observed that hemin itself destroyed hydrogen peroxide at the concentration employed in the growth medium, and the possible carry-over of hemin in the preparation of the cell-free extract could not be discounted. This consideration negated additional studies in this area.

Growth studies. Dobrogosz and Stone (1962*a*, *b*) demonstrated an enhanced aerobic utilization of glycerol only among strains of pediococci possessing catalase activity. When beef liver catalase was added to cultures of pediococci containing no catalase, glycerol was utilized to approximately the same extent as with the catalase-positive strains. Experiments were conducted to investigate an analogous advantage of the catalase-positive *S. faecalis* strains over catalase-negative strains. *S. faecalis* strains T91, P318, and two catalase-negative strains, FB82 and 10C1, were cultured aerobically and anaerobically with the use of graded concentrations of glucose, citrate, glycerol, and mannitol as energy sources. Unlike the pediococci, the possession of catalase appeared to offer no added advantage to the streptococci, as determined by their growth response and comparison with the catalase-negative strains under any of the test conditions.

Analysis of reaction end products. The possibility existed that a reaction between an end product of the organism's metabolism and hydrogen peroxide could account for the catalase-like activity of the *Streptococcus* strains under study. The incorporation of various α-keto acids in sterile agar medium and, after solidification, the development of the plates with 3% hydrogen peroxide produced an evolution of gas (CO_2) that could be confused easily with catalase activity. To rule out the possible involvement of an α-keto acid, both *Streptococcus* strains were grown on the surface of 50 ml of agar placed in a 500-ml filtration flask. After incubation for 24 hr, the air was evacuated and replaced with nitrogen, and 0.1 N barium hydroxide traps were connected in series to detect carbon dioxide evolution, 10 ml of 3% hydrogen peroxide were added with a syringe, and nitrogen was introduced slowly to facilitate CO_2 entrapment. Although catalase activity was observed when hydrogen peroxide was added, no CO_2 was detected in any of the cultures.

Additional evidence negating CO_2 as a reaction product was obtained manometrically by the inclusion and exclusion of 20% potassium hydroxide in the center well of the reaction vessels. The quantity of gas evolved when hydrogen peroxide was added to a cell suspension of *S. faecalis* T91 was virtually equivalent whether or not potassium hydroxide was present.

Oxygen evolution was detected by conducting the reaction in a three-arm reaction vessel. Cell-free extracts were placed in the main compartment and hydrogen peroxide was added to one side arm, pyrogallic acid (1 crystal) to another, and concentrated sodium hydroxide in the third. After flushing with helium and equilibrating, the hydrogen peroxide was tipped in. After a 10-min reaction period, the pyrogallic acid and sodium hydroxide were mixed and tipped in. Manometer readings were taken after equilibration, after the enzyme reaction with hydrogen peroxide, and after the addition of the pyrogallic acid-sodium hydroxide mixture. When the latter mixture was added, the reading on the manometer returned to the original figure obtained after equilibration and prior to the enzyme-hydrogen peroxide reac-

tion, thus indicating the absorption of the evolved oxygen by alkaline pyrogallol.

Detection of iron-porphyrin compounds. *S. faecalis* T91 and P318 were grown aerobically on agar plates of the basal medium with and without added iron, and were tested for iron-porphyrin compounds by the modified benzidine test of Deibel and Evans (1960). A negative test was obtained in all instances.

An attempt was made to detect iron-porphyrin compounds spectrophotometrically by employing a modification of the method of Moss (1957). Cells of *S. faecalis* T91 (2.5 g dry weight, grown aerobically in media supplemented with 0.2% ferric sulfate) and a strain of *Staphylococcus epidermidis* (0.53 g dry weight) were employed. The pyridine chromogens were examined in oxidized and reduced states (reduction by the addition of hydrosulfite) in a Beckman model DK recording spectrophotometer with the use of a pyridine-potassium hydroxide blank. Whereas a distinct absorption peak was observed in the 550-mμ band with the *Staphylococcus* strain, there was no indication of an absorption peak with the *Streptococcus* strain, although five times the weight of streptococcal cells were employed.

Enzyme purification. The protein from a crude cell-free extract (aerobically grown, iron-supplemented culture) was precipitated in an ice bath by the addition of solid ammonium sulfate. The bulk of the activity precipitated in the 50 to 100% saturated fraction. Nucleic acids were removed from this fraction by the addition of 6% protamine sulfate (pH 6.7; heated to 50 C; 0.28 ml was added per 100 mg of protein in the fraction). This treatment resulted in a 1.8-fold purification (Table 4). Further treatment of this fraction with increasing amounts of ammonium sulfate yielded a fraction (65 to 75% saturation) with a tenfold purification (Table 4). Samples of this fraction (20 mg of protein) were placed on a diethylaminoethyl (DEAE)-cellulose column (31 by 3 cm) and eluted with a phosphate buffer gradient (0.03 to 0.25 M sodium-potassium phosphate at pH 7.0). Catalase activity was eluted after 60 ml of buffer had passed through the column and continued to emerge until approximately 126 ml were collected. Fraction 7 (9.0-ml fractions were collected) yielded the highest specific activity (Table 4), and this fraction was employed in subsequent studies. The activity of the enzyme in this fraction was stable in that it did not diminish in a 3-week period when stored at 0 to 2 C.

TABLE 4. *Purification of the catalase-like enzyme produced by Streptococcus faecalis T91*

Fraction	Protein	Enzyme units[a]	Specific activity[b]	Purification[c]
	mg			
1 (Crude cell extract)	3,700	19,610	5.3	—
2 (0 to 50)[d]	1,800	840	0.5	—
3 (50 to 100)	2,000	18,870	9.4	1.8×
4 (50 to 100 + protamine)	1,500	14,900	9.9	1.9×
4a (0 to 50)[d]	450	—	—	—
4b (50 to 65)	610	2,400	3.9	—
4c (65 to 75)	200	10,300	51.5	10×
4d (75 to 90)	140	2,800	20.0	4×
4c7[e]	0.09	89.1	990	186×
4c8	1.215	204.0	168	32×
4c9	1.440	189.8	132	25×
4c10	0.990	140.2	142	27×
4c11	1.8	159.8	89	16×
4c12	2.25	130.0	58	11×

[a] One unit of enzyme activity is defined as that amount of enzyme releasing 1 μmole of oxygen from 26 μmoles of H_2O_2 in 5 min.

[b] Specific activity refers to the number of enzyme units per milligram of protein.

[c] Purification value refers to specific activity of sample per specific activity of crude extract.

[d] Figures refer to per cent ammonium sulfate saturation. Subfractions 4a to 4d were obtained from fraction 4.

[e] Figures refer to subfractions from a 20-mg sample of fraction 4c eluted from a diethylaminoethyl cellulose column. Fractions of 9 ml each were collected; therefore, 4c7 would be the seventh fraction. No activity was detected in fractions 4c1 to 4c6.

Although quite unsatisfactory from the standpoint of obtaining high yields of relatively pure enzyme, column chromatography on DEAE cellulose with the use of a buffered phosphate elution gradient proved to be superior to all other purification techniques employed. Other procedures attempted, including the use of Sephadex (G 50) and carboxymethyl-cellulose column chromatography, failed to resolve the activity as satisfactorily as did DEAE cellulose.

Optimal pH for catalase activity. Samples of the tenfold purified fraction (Table 4) were tested

for activity over a pH range of 4.5 to 9.0. The optimal pH was between 6 and 7, although some activity was noted at the extremes tested. These results paralleled those obtained by Delwiche (1961) with partially purified preparations from *P. cerevisiae*.

Effect of dialysis. Samples of the tenfold purified fractions were dialyzed (24 hr at 2 C) against four 1-liter quantities of deionized water and against four 1-liter quantities of 0.033 M phosphate buffer (pH 7.0) containing 0.0001% "Fe-specific" ethylenediametetraacetic acid (EDTA) (Kraft Chemical Co., Chicago, Ill.). The dialyzed samples were assayed for activity manometrically, with undialyzed samples used as controls. No loss of activity was observed in the dialyzed fractions.

Some characteristics of partially purified enzyme. The effect of heme poisons was determined manometrically on the partially purified enzyme (fraction 7 from DEAE-cellulose column); on a crude cell-free preparation; and, for comparative purposes, on beef liver catalase. Potassium cyanide or sodium azide (final concentration, 10^{-3} M in 0.033 M phosphate buffer, pH 7.0) was added to one side arm of the reaction vessel and hydrogen peroxide (26 μmoles) was added to the other arm. The heme poisons were tipped in first, and, after a 5-min equilibration period, the hydrogen peroxide was added. No loss of activity was noted with either of the heme poisons, although both inhibited the beef liver catalase completely. Atabrine was tested in an identical manner at a final concentration of 10^{-3} M; it also failed to inhibit the catalase-like activity.

DISCUSSION

Although the enhancement of catalase activity by aerobic growth of lactic acid bacteria has not been reported previously, Whittenbury (1960) observed a diminished "peroxide-splitting ability of a few heterofermentative lactic acid bacteria" when grown anaerobically. The effect of oxygen tension on the synthesis of various enzymes has been the subject of previous studies. The marked decrease in the cytochrome content of anaerobically grown *Pasteurella pestis* (Englesberg, Levy, and Gibor, 1954), *Bacillus coagulans* (Chaix and Flamens, 1953), and *B. cereus* (Shaeffer, 1952) exemplifies the effect of oxygen tension on the synthesis of these respiratory enzymes in some bacteria. More recently, Clayton (1960) observed a significant increase in the catalase content in aerobically grown cells of *Rhodopseudomonas spheroides* when compared with anaerobically grown cells. A similar effect of oxygen tension of enzyme synthesis was observed in strains of *S. faecalis* by Seeley and Vandemark (1951). These investigators observed a "peroxidase" that was formed only under high oxygen tension. Cultures grown anaerobically and subsequently induced to an aerobic metabolism did not contain this enzyme, and thus accumulated hydrogen peroxide.

The observation that supplementation of the growth medium with Fe^{+3}, Mn^{+2}, or Zn^{+2} increases catalase activity opens a number of avenues for further investigation. The requirement only under growth conditions infers a role in enzyme synthesis or, conceivably, the incorporation of the cation in the enzyme molecule itself. The inability to effect a diminished activity by dialyzing against the specific iron-chelating agent, EDTA, or to demonstrate a reduction of activity in cell-free preparations assayed with EDTA in the system, infers either the absence of iron or a strong bonding of the cation in the molecule. With the advent of more purified enzyme preparations, the quantitation of cation would aid in resolving its role in the overall system.

George (1948) and Wang (1955) reported the ability of certain complexes of ferric and ferrous salts to decompose hydrogen peroxide. In the present study, all attempts failed to demonstrate any ability of ferric sulfate, at the concentrations and pH employed, to decompose hydrogen peroxide when tested alone or in the uninoculated medium. Enzyme activity stimulated by ferric sulfate was destroyed by heating under exactly the same conditions as was the enzyme activity of cells grown in the absence of added iron.

The quantity of ferric sulfate required to produce optimal activity (5×10^{-5} g of ferric iron per ml) appears to be unusually high. It is possible, therefore, that the stimulation of catalase noted when certain divalent cations are added to the growth medium may be due to an unrecognized metal contaminant. However, ferric iron requirements of approximately the same magnitude have been reported previously. Melnykovych and Snell (1958) observed that optimal arginine decarboxylase activity in *Escherichia coli* occurred when 4.0 μmoles of

Fe^{+3} were added per liter of medium; and Hutner et al. (1950) observed a requirement of 2×10^{-6} g of iron per ml of medium for maximal growth of *Euglena gracilis*.

Recently, an iron-protein complex, ferredoxin, was shown to be functional in the electron transport system of certain cytochromeless anaerobic bacteria (Mortenson, Valentine, and Carnahan, 1962; Buchanan, Lovenberg, and Rabinowitz, 1963). Because of insufficient information regarding the streptococcal enzyme system, it is impossible to state whether a similar substance is associated with this enzyme.

As yet, in none of the studies associated with the catalase-like activity of the lactic acid bacteria has there been a definitive identification of either a coenzyme or the reactive group of the enzyme. In this study, as well as in previous studies (Deibel and Evans, 1960; Delwiche, 1961), the iron-porphyrin group characteristic of the classical catalase could not be detected, albeit the reaction end products are identical in both catalase systems. Further studies are needed regarding the nature of this unique enzyme, as well as its mechanism of action and kinetics.

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