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Title FLAVOR CONTROL IN DAIRY PRODUCTS AND BEER WITH
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The stability of diacetyl in fermented milk and the removal of diacetyl from beer were studied. A convenient method for the determination of diacetyl in beer, established by Owades and Jakovac, was modified and applied for flavor analyses of dairy products. Through this method, diacetyl in 12 samples could be determined simultaneously, facilitating the comparative study of diacetyl production and stability in milk during fermentation. A general parabolic curve for the synthesis and destruction of this compound was observed during the fermentation of milk by single or mixed-strain lactic streptococcus starter organisms at 21° C. The streptococci destroyed diacetyl by means of the enzyme diacetyl reductase. Lyophilized crude enzyme extracts of Aerobacter aerogenes was used as a source of diacetyl reductase and some characteristics of this enzyme were studied. The diacetyl reductase had an optimum activity at a pH between 6.0 and 7.0, while its activity was remarkably inhibited at pH values below

5.5. The crude enzyme preparation was quite stable during storage at -20°C.

The rapid destruction of diacetyl in milk at 21°C could be prevented by cooling the culture promptly (2°C) after the maximum production of diacetyl. Apparent chemical conversion of precursor to diacetyl was also observed at this low temperature during storage. About 7.5 ppm of diacetyl was found in cottage cheese dressed with cultured cream prepared in this manner using Streptococcus diacetilactis and held at 5°C for 20 days; only a trace amount (0.2 ppm) of diacetyl was found when the cheese was dressed with non-cultured cream. Another method for the enhancement of diacetyl in fermented milk was developed: heated (121°C for 13 min) nonfat milk (100 ml) cooled to 25°C was treated for 20 minutes with 0.03 percent hydrogen peroxide, and was then exposed to sufficient concentration of catalase to destroy the oxidant. The milk, in tightly capped containers, was inoculated with one percent of a mixed-strain starter culture containing S. diacetilactis and held at 21°C. Diacetyl level rose rapidly to at least 14 ppm within 15 hours and decreased slowly to 9 ppm upon holding for eight days at 21°C. Nonperoxide-treated controls produced less total diacetyl (5 to 8 ppm), which was rapidly reduced within 24 hours to less than 2 ppm. Also, the amounts of diacetyl desired in the finished product could be controlled by adjusting the concentration of hydrogen peroxide. Reduction of the level of

hydrogen peroxide from 0.03 percent to 0.015 percent, lowered diacetyl synthesis, and the stability of diacetyl in culture was also reduced to about one-half. The reduced effect on the stability of diacetyl at 21°C when milk was treated with lesser amounts of hydrogen peroxide was remedied by combining the cooling process with this treatment. Diacetyl level in a mixed-strain culture held for five days at 21°C was about 3 ppm when the milk was treated with 0.015 percent of hydrogen peroxide. However, more than 5 ppm of diacetyl was detected in the culture cooled to 2°C and held for the same period of time after the development of 0.85 percent acid.

Removal of diacetyl, which is undesirable in alcoholic beverages, was attempted by use of diacetyl reductase. It was found that diacetyl could be removed from beer when high concentrations of diacetyl reductase and reduced pyridine nucleotide were applied. The amounts of these two components required could be reduced by coupling the diacetyl reductase system to the alcohol dehydrogenase system, but the levels of enzyme and cofactor needed were still too high for practical use. The reason for the low activity of the coupled system in the intact beer was traced to the low pH of the beer (4.3) and this was the limiting factor in the application of diacetyl reductase.

FLAVOR CONTROL IN DAIRY PRODUCTS AND BEER
WITH SPECIAL REFERENCE TO DIACETYL

by

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FLAVOR CONTROL IN DAIRY PRODUCTS AND BEER WITH SPECIAL REFERENCE TO DIACETYL

INTRODUCTION

The consumption of a food is largely influenced by its flavor which is a combined effect of odor, taste and touch. Among the substances which might be responsible for the desirable flavor of dairy products, diacetyl has been considered as the most important substance since the early period of this century. Diacetyl is a volatile compound and contributes to the fine pleasant flavor of cottage cheese, butter milk, cultured sour cream, and other fermented dairy products mainly through odor. With progress in dairy microbiology, the origin of diacetyl in milk, the organisms responsible for the synthesis of this compound as well as its mechanism of synthesis have become more clear. Diacetyl is produced in milk primarily during the fermentation by aroma bacteria of citric acid. However, the same aroma bacteria as well as psychrophilic spoilage organisms have a capacity to decompose the synthesized diacetyl resulting in loss of the desired flavor. Many investigators have been challenged to study the problem of this flavor loss in dairy products, but the basic cause has remained unknown. In the alcoholic fermentation industry, on the other hand, the presence of diacetyl causes a serious off-flavor and research workers have studied this problem in recent years,

especially as it relates to beer.

The present study was primarily concerned with the stabilization of diacetyl to improve flavor in fermented milk and related dairy products. In addition, a study on the application of diacetyl reductase to remove diacetyl from beer was made.

HISTORICAL REVIEW

Diacetyl Production by Aroma Bacteria

It is well-known today that diacetyl is a major contributor to the desirable flavor of most cultured dairy products. This knowledge originated from the first detection of diacetyl by Schmalfuss (58) in 1928. He detected diacetyl by the sense of smell in a milk culture of a rod-shaped lactic acid organism and, through analysis, confirmed the identification. In the next year, van Niel, Kluyver and Derx (68) reported studies on the relationship of acetylmethylcarbinol and diacetyl to the aroma of butter, and concluded that diacetyl is either responsible for the aroma of butter or is the principal component of the aroma fraction. These findings have since been substantiated by many workers, and though some other chemical compounds have also been reported to be important for desirable flavor, diacetyl still occupies the central position among them. These other compounds are organic acids such as lactic, acetic, and propionic as well as carbon dioxide (21). Acetaldehyde also has been recently included in this category of desirable components in normal culture flavor if present in low concentration, especially in proper ratio to diacetyl (34, 35).

The study of organisms which are responsible for flavor production in mixed-strain starter cultures was initiated even before

diacetyl was identified in the cultures. In 1919, Hammer and Bailey (20) isolated flavor producing organisms from a mixed culture of lactic acid-producing bacteria. Three years later, Hammer (19) reported taxonomic results on these associative bacteria which he designated Streptococcus citrovorus and Streptococcus paracitrovorus. These species could produce a high volatile acidity when they were grown with lactic acid producing bacteria such as Streptococcus lactis. The addition of either citric acid or lactic acid to pure cultures of either of these species was necessary for the production of volatile acidity. These organisms therefore came to be known generally as aroma or flavor bacteria. They were used in association with lactic acid-producing bacteria to manufacture ripened cream butter which possessed superior flavor and aroma.

These organisms were once included in the genus Betacoccus as proposed by Orla-Jensen and his co-workers (45) and later, were unified to one species Betacoccus cremoris by Knudsen and Sorensen (32) in 1929. The next year, however, Hucker and Pederson (27) re-separated these organisms into two species and placed them in the genus Leuconostoc. S. paracitrovorus was designated Leuconostoc dextranicum and S. citrovorus was renamed Leuconostoc citrovorus, which is now called Leuconostoc citrovorum.

An aroma bacterium different from Leuconostoc organisms was isolated and named Streptococcus diacetilactis in 1936 by

Matuszewski and his co-workers (37). This organism can produce both diacetyl and lactic acid in milk; therefore, the association with other acid-producing bacteria is not essential for flavor production.

Citric Acid as a Source of Diacetyl in Milk

In 1920, Hammer (18) observed that aroma bacteria produced more volatile acids in milk fortified with citrate. From this observation, attention has been directed to citrate, which is a common constituent of milk at an average level of about 0.2 percent. Thus the citric acid fermentation by lactic acid streptococci and Leuconostoc organisms has been widely studied.

Brewer (4) demonstrated that L. dextranicum could not attack citrate in the absence of sugar but would in presence of catalytic amounts of glucose and lactose. Slade and Werkman (65) found that cell suspensions of L. dextranicum, which was grown in the presence of citrate plus lactose, were able to ferment citric acid in the absence of carbohydrate. Storgards (66) found that the products of glucose fermentation by citric acid-fermenting bacteria from butter cultures were carbon dioxide, ethyl alcohol, formic, acetic, and lactic acids. Acetylmethylcarbinol, diacetyl and 2,3-butylene glycol were not found. In the fermentation of solutions containing both glucose and citric acid, aroma substances were found only in the presence of calcium ion. Federov and Kruglova (16) traced the source of flavor and aroma compounds to citric acid. They found that four strains of S. diacetylactis produced diacetyl and acetoin from citric

acid but not from lactose.

On the other hand, there are some reports in literature to suggest that citrate is not the source of the flavor compounds. For example, Virtanen et al. (69) reported that the sugar, not citric acid, is the essential substance for aroma production. Acetylmethylcarbinol was formed from glucose in the presence of methylene blue or quinone, but not in the absence of a suitable hydrogen acceptor. Citric acid did not give acetylmethylcarbinol even in the presence of methylene blue. Coppens (9) also concluded in his report that lactose was the main source of flavor and that citric acid played some auxilliary role.

Anderson (1), however, found that both lactose and citric acid were fermented to diacetyl and acetoin by L. citrovorum and S. diacetylactis. This finding was supported by Mizuno and Jezeski (41). They studied the mechanism of acetylmethylcarbinol formation from radioactive isotopes of glucose, citric acid and pyruvic acid in mixed-strain starter cultures containing Leuconostoc. From this study they concluded that the origin of the carbon skeleton for acetoin was either glucose or citrate, each of which was metabolized to a common precursor, namely pyruvate.

The recent results by the following investigators De Man and Pette (13), Rushing and Senn (56) and Mizuno and Jezeski (40) demonstrated that citrate-derived pyruvate was much more available

for diacetyl production than pyruvate resulting from the catabolism of glucose, lactose, or other fermentable sugars. Harvey and Collins (23) gave a possible explanation for the failure of aroma bacteria to produce acetoin from lactose in the absence of citrate. In the anaerobic metabolism of these organisms, the reduced pyridine nucleotide liberated from the oxidation of glyceraldehyde-3-phosphate to 1, 3-diphosphoglycerate, has to be reoxidized by coupling the reaction to the reduction of pyruvate to lactic acid. Therefore, no pyruvate is available for diacetyl and acetoin synthesis if these organisms are grown on carbohydrate alone, but if they are grown on carbohydrate plus citrate, excess pyruvate is produced to permit synthesis of these compounds. Busse and Kandler (7) also agreed with this explanation.

On the basis of the above citrate origin theory, Sandine and his co-workers (62) established the most recent pathways for conversion of citric acid to diacetyl, acetylmethylcarbinol, and 2, 3-butanediol by S. diacetylactis. From the study using the resting cell and cell-free extracts of this organism, they found that S. diacetylactis produced diacetyl from citrate with the sequential production of oxaloacetate, pyruvate, and α - acetolactate as intermediates. They also proved the presence of diacetyl reductase, which reduces diacetyl to acetoin irreversibly, and a reversible 2, 3-butanediol dehydrogenase in this organism.

Factors Affecting Diacetyl Production in Milk

Among the factors which affect diacetyl production in milk, the effect of pH, which is directly related to the symbiosis between aroma and lactic acid-producing bacteria in mixed-strain starter cultures, has been studied most widely. In 1896, Weigmann (71) obtained fine-flavored butter by manufacturing it from cream ripened with acid and aroma-producing bacteria. This was the first suggestion for combining these two types of organisms for better flavor. Later investigations threw much light on the mechanism of this combination effect.

From a single strain of S. citrovorus, Michaelian et al. (38) prepared fine-flavored cultures for use in butter manufacture by lowering pH of the milk using citric acid and sulfuric acid after 24 hours of incubation at 21° C. There was usually no acetoin or diacetyl present before the addition of acid to the milk, and the most rapid production of aroma occurred upon further incubation for 24 hours at pH 4.0 to 4.3. Ruments (55) found that when citric acid was added to a milk culture of S. citrovorus, the amount of acetoin and diacetyl formed could be considerably increased. The optimum pH for the formation of diacetyl was 4.3, and it made no difference whether citric acid or lactic acid was used to acidify the milk. Wiley et al. (74) reported that when Betacoccus organisms were grown

in milk heated to 93° C for one hour, no diacetyl was produced even after incubation for 100 hours. However, when the cells were grown in the same milk acidified to pH 4.2, they produced and destroyed diacetyl at a rapid rate. Cox (10) also found that the rate of diacetyl synthesis and degradation was largely dependent on pH; milk was inoculated with Betacoccus organisms at the rate of five percent and incubated at 21° C with slow, constant additions of lactic acid. By this procedure, maximum diacetyl level could be reached sooner than when the pH level was adjusted to pH 4.4 at the beginning of incubation.

The pH favorable for the accumulation of acetylmethylcarbinol and diacetyl in single strain culture of an aroma bacterium could be established by growing it with lactic acid-producing bacteria such as S. lactis as pointed out by Michaelian and his co-workers (39). According to Foster et al. (17, p. 291), many strains of Leuconostoc produce comparatively little lactic acid from lactose, and, therefore, fail to lower the pH in the culture to a level conducive to production of maximum amounts of the flavor and aroma compounds. Such a lack of sufficient acid production by Leuconostoc organisms resulting in poor aroma formation may be remedied by either acidifying the milk after their maximum growth or associating with acid-producing bacteria.

A similar beneficial effect of low pH on flavor production was

shown also in S. diacetylactis strains by Seitz et al. (60). The authors tested 16 different strains of S. diacetylactis and found greater quantities of diacetyl when the pH was adjusted to 4.3 with phosphoric acid after logarithmic growth of these organisms followed by an additional 18 hours of incubation.

Recent work by Harvey and Collins (24) has led to a better understanding of the mechanism of the pH effect on the biosynthesis of aroma compounds. They found that the rates of citrate uptake by intact cells of S. diacetylactis and L. citrovorum increased rapidly below pH 6.0. They also demonstrated the presence of a citrate transport system in S. diacetylactis which was similar to the β - galactoside permease of Escherichia coli.

There are only a few reports concerning factors other than pH which influence diacetyl production in milk. Seitz (59) found that the incubation time at 22°C had a great effect on diacetyl production. Diacetyl increased through 20 to 24 hours, followed by a significant destruction by prolonged incubation. To study the effect of the heat treatment given to the culture milk on diacetyl production, he used two types of flavor organisms, Leuconostoc and S. diacetylactis. Inhibitory effects were observed when both excessive heat treatment (121°C for 25 minutes) and improper pasteurization (62°C for 30 minutes) were used. Heat treatment of 121°C for 12 minutes showed the maximum production of diacetyl. Leuconostoc organisms were slightly

more sensitive to the heat treatment than S. diacetylactis.

Contamination by spoilage or psychrophilic bacteria causes off-flavor in cultured products. Elliker and Horall (15) observed that samples of butter contaminated with Pseudomonas putrefaciens displayed a marked loss of aroma during storage. Later, Elliker (14) demonstrated the ability of a number of types of bacteria to destroy diacetyl and also indicated that diacetyl was reduced to acetylmethylcarbinol and 2, 3-butanediol by the action of P. putrefaciens. In 1953, Parker and Elliker (50) reported that cultures of P. viscosa and P. fragi isolated from gelatinous cottage cheese were capable of rapidly reducing almost all diacetyl present in cottage cheese or in milk. Recently, Seitz et al. (61) demonstrated the presence of diacetyl reductase in these psychrophilic organisms, thus providing an explanation for the flavor loss in contaminated dairy products.

Diacetyl as an Undesirable Flavor Component in Beer

Though diacetyl is desirable when present in suitable concentration in certain dairy products, its presence causes objectionable flavor defects in beer. The off-taste in beer caused by this substance has been usually called sarcina-sick, or diacetyl or sarcina aroma. Earlier investigators have endeavored to eliminate this substance from beer without much success.

According to Claussen (8), diacetyl in beer is produced by beer

cocci belonging to genus Pediococcus. Shimwell and Kirkpatrick (63), after a comparative study, concluded that the so-called "beer-sarcinae" or "beer pediococci" belong to genus Streptococcus. Siromalainen and Jannes (64) found that brewer's yeast also produced acetylmethylcarbinol and 2, 3-butylene glycol during the fermentation of sugar. They stated that the reason diacetyl was not produced during the sugar fermentation by yeast was due to the immediate reduction of the diacetyl formed. Recently, Burger et al. (5) reported that Lactobacillus pastorianus, which is often found in beer at the lagering stage, also produced diacetyl. Furthermore, these authors concluded in their report that diacetyl can be formed non-microbiologically in beer, especially if the beverage is exposed to air for an unduly prolonged period at certain stages of processing.

The diacetyl concentration found in normal tasting beer as reported in the literature varies widely. West (72) reported that normal tasting beers had diacetyl contents ranging from 0.20 to 0.46 ppm, and that the diacetyl becomes perceptible when it reaches about 0.35 ppm. However, Burger et al. (5) mentioned that some of the beer on the market today was so mild that amounts of diacetyl even somewhat less than 0.35 ppm could be organoleptically detected. They stated in the same report that the diacetyl content in beer of normal taste was usually about 0.2 ppm.

Among the various attempts to remove diacetyl from beer, the

use of yeast cells is noteworthy. Burger et al. (5) reported that the undesirable diacetyl flavor may be removed from a beer by fermenting it with about one-half its volume of fresh wort and with yeast at the normal rate. Kato and Nishikawa (31) also found that addition of fresh yeast to beer to which diacetyl had been added was effective in its elimination regardless of yeast species. Recently Lagomarcino and Akin (33) studied factors affecting the removal of diacetyl from beer by added cells of Saccharomyces cerevisiae. They found that the diacetyl removal rate increased with increased temperature and yeast concentration, and was faster when diacetyl concentration was greater.

Burger et al. (6) prepared crude cell-free extracts of yeasts by various methods--namely plasmolysis followed by autolysis using chemical agents such as toluene, ethyl acetate, etc., and by a process of alternate freezing and thawing followed by centrifugation--and used various amounts of such preparations to remove diacetyl from beer. All such attempts failed, while whole cells were found to effect the desired destruction. From this the authors concluded that the diacetyl destroying enzyme was tightly bound to the yeast cell material and hence was not found in the cell-free preparations.

EXPERIMENTAL METHODS

Owades and Jakovac Method for Diacetyl Determination in Mixed-strain Starters

Cultures Used

Single-strain organisms were available from the stock culture collection in the Department of Microbiology, Oregon State University. These were S. lactis strains E and C2, S. cremoris strains 1 and C3, and S. diacetylactis strains 26-2 and 18-16. Mixed-strain starter cultures referred to in Table 6 as E, F, G, and H were obtained from commercial sources. All cultures were maintained in 10 ml of sterile (121°C for 12 minutes) inhibitor-free ten percent nonfat milk by transfer of one percent inoculum every other day and incubation for 18 hours at 21°C.

Owades and Jakovac Method

The Owades and Jakovac method which was originally designed to determine diacetyl in beer was modified for the use in dairy products. Twelve separate determinations could be run simultaneously through the use of gassing manifold as shown in Figure 1.

Apparatus. Following the original description by Owades and Jakovac (46, 47) an apparatus for diacetyl determination in dairy

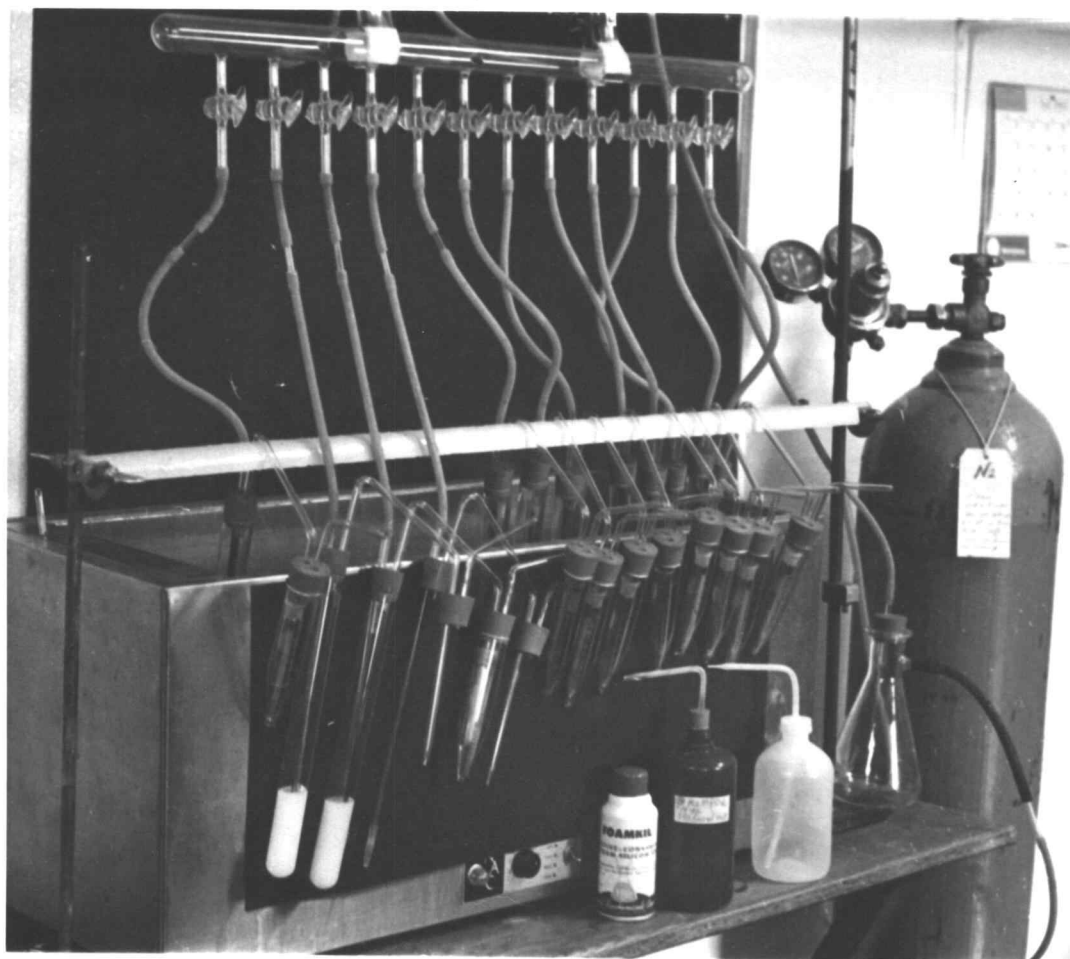


Figure 1. Apparatus used for diacetyl determinations, showing gassing manifold (top), reaction, and trapping vessels.

products was constructed with a few modifications. A large test tube (25 by 250 mm--Corning No. 9820), shown outside the water bath on Figure 1, was fitted with a two-hole No. 5 soft rubber stopper, one hole occupied by a 1.1 ml milk dilution pipet running to the bottom of the tube. The top of this pipet was connected to the nitrogen supply as shown. The other hole in the No. 5 stopper was fitted with an inverted U-shaped tube which protruded only 1 cm or so below the base of the stopper. This tube led to a two-hole No. 6 stopper mounted in a 50-ml centrifuge tube (Corning No. 8100) which contained a 12-ml centrifuge tube (Kimax No. 54199) cushioned on a small piece of rubber about 0.5 mm square. The U-tube extended through the rubber stopper to the bottom of the inner 12-ml centrifuge tube, which contained 1.0 ml of buffered hydroxylamine. The second hole of the No. 6 stopper was occupied by a short piece of angle tubing, as shown in Figure 1.

Reagents. The reagents used were the same as those described by Prill and Hammer (54) and Owades and Jakovac (47) and their composition is as follows:

1. Buffered hydroxylamine, prepared by mixing K_2HPO_4 (33 g of K_2HPO_4 in 100 ml of distilled water), $NH_2OH \cdot HCl$ (11.0 g made to 250 ml with distilled water), sodium acetate (35.0 g made up to 100 ml with distilled water) 2:4:1.

2. Acetone-phosphate, prepared by dissolving 29.0 g of

K_2HPO_4 (38 g of $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$) in distilled water followed by addition of 40 ml of pure acetone and dilution to 200 ml with distilled water; store in refrigerator.

3. Alkaline tartrate, prepared by mixing saturated potassium sodium tartrate (100 g/150 ml of distilled water) with concentrated ammonium hydroxide 22:3.

4. Ferrous sulfate prepared frequently (when slight yellow color begins to appear) by dissolving 5.0 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in 100 ml of one percent sulfuric acid.

Procedure. Twenty milliliters of the 18-hour culture to be tested were placed in the test tube and Foamkil (Nutritional Biochemicals Corp., Cleveland, Ohio) added from a pressurized spray can. The reaction unit was assembled and suspended over a wooden rod mounted over the water bath so that the culture-containing tube was immersed in the water (65°C) while the hydroxylamine trap was outside the bath. Gas from the nitrogen tank was allowed to enter the system by successive passage through a 20-liter carboy, a 500-ml suction flask, the manifold, the sample-containing tube, the hydroxylamine trap, and finally out the angle vent tube. A gas flow to produce about five to seven bubbles per second was used, but variation in the rate did not alter results; excessive bubbling was avoided. Owades and Jakovac (47) recommend a gas flow of about 100 to 150 ml per minute.

After gassing for 1.5 hours, the hydroxylamine trap was disconnected (with gas still flowing) and the tip of the inverted U-tube rinsed into the trap with a few drops of 33 percent K_2HPO_4 ; a few drops of rinse also were added to the inside of the other end of the U-tube and forced into the hydroxylamine trap by air pressure from an empty squeeze bottle.

The 12-ml centrifuge tubes then were immersed in a 75° C water bath for ten minutes, removed and, while still warm, diluted with 0.5 ml of the acetone-phosphate solution and mixed. After cooling, 1.5 ml of alkaline tartrate were added, followed by mixing. Then 0.1 ml of ferrous sulfate was added and mixed immediately. The volume was adjusted to 5.0 ml with 33 percent K_2HPO_4 and absorbancy of the pink color read at 530 m μ , using a Bausch and Lomb Spectronic 20 colorimeter. The concentration of diacetyl was determined by comparison with a standard curve of dimethyl glyoxime. Blanks used to zero the colorimeter were sterile, uncultured nonfat milk treated exactly as the samples being tested for diacetyl; such blanks gave absorbancy readings of 0.01 to 0.02 when read against water reagent blanks at 530 m μ . Dimethyl glyoxime (K and K Laboratories) was used as the standard rather than diacetyl to avoid frequent distillation of diacetyl; dimethyl glyoxime is the compound actually measured in the colorimeter as a result of a reaction between diacetyl and hydroxylamine. The solution of glyoxime was prepared

as described by Prill and Hammer (54), so that each milliliter was equivalent to 100 μ g of diacetyl.

Comparison with Two Other Methods

For the comparison with the Owades and Jakovac method, diacetyl determination on single- and mixed-strain lactic streptococcus cultures were made using the procedure described by Prill and Hammer (54) and also the method described by Pien et al. (53), as modified by Elliker (14). Reagent blank used to zero the colorimeter with these two methods was sterile, uncultured nonfat milk, steam-distilled and otherwise treated exactly as the samples being analyzed. During the Pien method, distillate (50 ml) was collected in a volumetric flask from 20 ml of sample. When distilled water was used to zero the colorimeter, it was found that blanks from sterile milk gave absorbancy readings of about 0.3 at 425 m μ , due to heat-induced production of carbonyl compounds which reacted under acidic conditions with the 3-3' diaminobenzidine tetrahydrochloride (K and K Laboratories); distilled milk previously heated at 62° C for 30 minutes gave absorbancy readings of about 0.1 under the same conditions. The Prill and Hammer method was used as originally described (54), except that the instrument was not flushed with CO₂.

Diacetyl Production and Destruction Patterns in Mixed-strain Starters

Cultures Used

The single strain S. diacetilactis 18-16 was available from the stock culture collection in the Department of Microbiology, Oregon State University, and the mixed-strain starter cultures A, B, C, D, E and G were obtained from commercial sources. All cultures were maintained in 10 ml of sterile ten percent nonfat milk by transfer of one percent inoculum every other day and incubation for 18 hours at 21°C.

Typical Pattern

One hundred ml of ten percent nonfat sterile milk in screw-capped bottles was inoculated with 1 ml inoculum of mixed-strain culture E and was incubated at 21°C. The inoculum was prepared by the incubation for 18 hours under the same cultural conditions. The amount of diacetyl in the culture was determined at specific times by the modified Owades and Jakovac method (49) using 20 ml samples from different bottles.

Cultural Variations

The same cultural and diacetyl determination methods as

described above were applied except using different cultures. One single strain S. diacetylactis 18-16 and three mixed-strain cultures, C, D and G, were used for this experiment.

Temperature Effect

Two mixed-strain cultures (A and B) containing known organisms were selected for this experiment. Culture A contained S. cremoris and Leuconostoc strains, and Culture B contained S. cremoris and S. diacetylactis strains. In order to avoid strain-dominance and other phenomenon which might shift the proportions of the component strains with successional transfers, the original commercial lyophilized culture was directly used as the inoculum. Mixed-strain culture powder (200 mg) was seeded in one gallon of nine percent nonfat milk which was steamed for one hour in a stainless steel can with a tight cover, and incubated at two different temperatures, 21° C and 32° C. The diacetyl content was determined periodically in 20 ml samples drawn from the same can.

Studies on Diacetyl Reductase

Enzyme Preparation

Aerobacter aerogenes ATCC 8724, from the stock culture collection in the Department of Microbiology, Oregon State University,

was grown from a one percent inoculum in 18 liters of sterile citrate broth for 24 hours at 30° C. The citrate broth (57) contained the following: tryptone, one percent; glucose, one percent; sodium citrate, two percent; yeast extract, 0.5 percent; dibasic potassium phosphate, 0.1 percent; and magnesium sulfate, 0.1 percent. The pH was adjusted to 7.0 with hydrochloric acid.

Following growth, the cells were harvested with the use of a Sharples centrifuge equipped with a cooling coil to prevent the temperature of the collecting bowl from exceeding 10° C. The cells were resuspended in 0.85 percent sodium chloride, washed three times, and resuspended in 0.1M potassium phosphate buffer at pH 7.0.

Cell-free extracts were prepared by disrupting the cells in a Raytheon 10KC sonic oscillator for 15 minutes. Cell debris was removed by centrifugation at 12,000 rpm for two hours in a refrigerated centrifuge set at 2° C. The supernatant was dialyzed against three four-liter changes of cold (2° C) distilled water, each dialysis lasting eight hours. The crude enzyme was then lyophilized and stored in a deepfreeze until used. The yield of lyophilized enzyme from 18 liters of culture by this procedure was usually about 3 g and the average enzyme units per mg of solids were around 35,000.

Enzyme Kinetics

A continuous recording spectrophotometer, Cary Model 11, was

used to measure the activity of diacetyl reductase by following changes in absorbancy at 340 m μ caused by oxidation of DPNH. The reactions were initiated by the addition of diacetyl to solutions containing diacetyl reductase, DPNH, and buffer. The concentration of the crude enzyme was adjusted to the range where no DPNH oxidation occurred before the addition of the substrate, diacetyl (Figure 8). A typical experimental set-up is shown in Table 1. After adjusting the blank (enzyme alone) to 100 percent transmission, the initial optical density after the addition of DPNH (Plus DPNH) was recorded. One-tenth ml of standard diacetyl solution (860 μ g/ml) was then added to cuvet containing enzyme and DPNH (Complete) with quick agitation, and the oxidation of DPNH was initiated. Time required for 50 percent reduction of the initial optical density was used as the index of the enzyme activity. All assays were carried out at room temperature (25° C).

Table 1. A Typical Experimental Design for Assay of Diacetyl Reductase

	Blank	Plus DPNH	Complete
	ml	ml	ml
Enzyme (5 mg/ml)	0.1	0.1	0.1
DPNH (2 mg/ml)	-	0.2	0.2
Diacetyl (860 μ g/ml)	-	-	0.1
Buffer (0.1 M KH ₂ PO ₄ , pH 7.0)	2.9	2.6	2.6

Unit and Specific Activity of Enzyme

The reaction time in seconds (T) obtained from the above procedure was used for the calculation of enzyme units present. One unit of enzyme was defined as the amount of enzyme which caused a 50 percent reduction of absorbancy when $1/T = 10^{-6}$. The following example will explain a typical calculation of unit and specific activity of diacetyl reductase.

Enzyme concentration in reaction mixture = 0.5 mg

Time for 1/2 initial absorbancy = 100 seconds

$$1/T \div 10^{-6} = 10,000 \text{ units}$$

$$10,000 \div 0.5 = 20,000 \text{ units per mg}$$

Storage Temperature Effect

Lyophilized enzyme was stored at various temperatures in screw-capped test tubes for eight weeks. In order to examine the stability, the units per mg of enzyme were determined periodically.

pH Effect

Effect on Diacetyl Reductase. The cuvet was filled with 0.5 mg of enzyme, 0.4 mg of DPNH, and 0.1 M potassium phosphate buffer whose pH was adjusted to various levels using potassium hydroxide and phosphoric acid solutions. After recording the initial absorbancy,

0.1 ml of 860 $\mu\text{g/ml}$ standard diacetyl solution was added and the decrease in absorbancy in two minutes was read.

Effect on DPNH. 0.4 mg of DPNH was added to 3 ml of 0.1 M potassium phosphate buffer having different pH values and its absorbancy at 340 $\text{m}\mu$ was followed for two minutes.

Effect of Cooling on Diacetyl Stability in Mixed-strain Starters and Dressed Cottage Cheese

Effect of Cooling on Mixed-strain Starters

Mixed-strain starters, E and G, obtained from commercial suppliers were used. One ml of precultured starter was inoculated in 100 ml of ten percent nonfat milk in screw-capped bottles and incubated at 21° C. Part of the bottles was transferred to a refrigerator (2° C) after certain intervals of time. Diacetyl contents in bottles under both temperatures were determined periodically.

Cooling Effect on Dressed Cottage Cheese

Cottage cheese creamed with cultured dressing was prepared on a commercial scale as follows: regular dressing cream (12 percent butterfat) was incubated at 21° C with two percent inoculum of S. diacetylactis 18-16 for ten hours. The cultured cream was mixed with regular dressing cream at the ratio of 4:7 to obtain the so-called cultured dressing. Three parts of cultured dressing was mixed with

four parts of dry cottage cheese curd.

Ten pounds of the dressed cottage cheese were divided into one pound portions and packed in pint-containers. Five of the packages were stored in a cold room at 5°C, and the remaining were packed in wet ice to maintain the temperature constantly at 0°C. To serve as a control, another five containers of cottage cheese dressed with non-cultured cream were also held in the cold room at 5°C.

The diacetyl content in each of these samples during one month of storage was determined periodically using the Owades and Jakovac apparatus described earlier. Samples (150 gm) from each pint-container were mixed with equal amounts of distilled water and blended for a few minutes in a Waring blender. Twenty ml of the cottage cheese homogenate was used for the determination.

Effect of Hydrogen Peroxide-Catalase Treatment on Diacetyl Stability in Single and Mixed-strain Starters

Cultures Used

S. diacetylactis 18-16 from the stock culture collection in the Department of Microbiology, Oregon State University, and mixed-strain culture E obtained from a commercial supplier were selected for this study. Culture E is known to contain S. cremoris, S. diacetylactis and L. citrovorum strains (25).

Hydrogen Peroxide-Catalase Treatment of Milk

The solutions of hydrogen peroxide and catalase used were obtained from Marschall Dairy Laboratory, Inc., Madison, Wisconsin.

One hundred ml of ten percent nonfat milk in a screw-capped bottle were autoclaved for 12 minutes at 121°C and cooled to the room temperature. Then, the hydrogen peroxide was added to the milk at the rate of 0.01-0.03 percent based on the weight of the milk. After about 20 minutes, a sufficient amount of catalase was added and mixed thoroughly to decompose the hydrogen peroxide in the milk.

The amount of the catalase required for the complete decomposition of the hydrogen peroxide in a few minutes is shown in Table 2. From the results, it was concluded that 0.002 percent or 0.004 percent of catalase was sufficient for the complete decomposition of hydrogen peroxide added to the milk at concentrations of 0.015 percent or 0.03 percent respectively.

Table 2. The Amount of Catalase Required for the Decomposition of Hydrogen Peroxide. Ten ml of the hydrogen peroxide-catalase treated milk were transferred into a test tube and five drops of freshly prepared 40 percent potassium iodide solution were added. The symbol + indicates a yellow discoloration due to the presense of the hydrogen peroxide and the symbol - (no color change) indicates no oxidant remains.

Catalase added %	Reaction with KI in milk treated with H ₂ O ₂ of	
	0.015 %	0.03%
0.000	+++	+++++
0.001	+	++
0.002	-	+
0.004	-	-
0.006	-	-

Combined Effect of Hydrogen Peroxide-Catalase Treatment and Cooling on Diacetyl Stability

The milk treated with various amounts of hydrogen peroxide-catalase was inoculated with one percent or two percent of mixed-strain culture E and incubated at 21°C. When the titrable acidity of the milk reached 0.85 percent, one set of the bottles was transferred to a refrigerator while the other set was held at the incubation temperature (21°C). The diacetyl content in the bottles stored for five days under the two different temperatures were determined at various intervals.

Enzymatic Removal of Diacetyl from Beer

Materials Used

Canned beers of several different commercial brands were checked for their pH levels and one product which seemed to have a relatively uniform pH of about 4.3 was selected for the experiments. Partially purified A. aerogenes diacetyl reductase having 35,000 units per mg of solids was used. Reduced diphosphopyridine nucleotide (DPNH) and alcohol dehydrogenase obtained from Sigma Chemical Co., St. Louis, Missouri, also were used for this study.

Effect of Diacetyl Reductase and DPNH

The initial amounts of diacetyl in the beer samples were

raised to levels ranging from 0.10 to 0.50 ppm by adding synthetic diacetyl. The fortified beer (20 ml) was pipetted into sample tubes of the Owades and Jakovac apparatus containing different amounts of diacetyl reductase and DPNH. After mixing, these tubes were tightly stoppered, and allowed to stand for three hours at room temperature. Diacetyl levels in these tubes were then determined by the modified Owades and Jakovac method.

Effect of Coupling with Alcohol Dehydrogenase

The regeneration of DPNH from oxidized DPN by coupling to the alcohol dehydrogenase system was attempted by following the optical density at 340 m μ using the continuous recording spectrophotometer. Reduced cofactor (0.4 mg) was added along with 0.5 mg of diacetyl reductase to the cuvet containing 0.1 M phosphate buffer (pH 7.0). The reaction was started by the addition of 86 μ g of diacetyl and allowed to proceed until the cofactor was completely oxidized. When the absorbancy reached the minimum level, 0.1 ml of 95 percent ethanol and 16,000 units of alcohol dehydrogenase were added to initiate the increase in absorbancy indicating the regeneration of DPNH. The effect of the coupled system on the reduction of diacetyl in beer at low temperatures was also examined by adding the components of the system at various levels to beer which was previously fortified with diacetyl up to 4.0 ppm. These mixtures were

held for 12 hours at 2° C before the determination of the final diacetyl levels.

Effect of pH

The activity of diacetyl reductase as well as the effect of coupling with the alcohol dehydrogenase system in neutral buffer solution was determined by comparing the added diacetyl levels before and after holding the reaction mixture for 12 hours at 2° C. Similar experiments were performed using beer in which pH values were adjusted to different levels by adding potassium hydroxide to beer. A pH of 5.5 in beer was selected for studying the effects of reaction time and ratio of the components of the coupled system on the reduction of diacetyl.

RESULTS

Owades and Jakovac Method for Diacetyl Determination in Mixed-strain Starters

Spectra of Diacetyl Color Complex Compounds

Figure 2 shows the spectrum of the diacetyl-colored complex recovered from milk using the Owades and Jakovac method; diacetyl recovered from water showed an identical absorption spectrum. Maximum absorption occurred at 530 m μ and it also may be seen that the control milk gave negligible absorption at this wavelength. The absorption spectrum of the color complex of diacetyl and 3-3' diamino-4,4'-dihydroxydiphenylmethane tetrahydrochloride is shown in Figure 3.

Standard Curve

Figure 4 shows a typical standard curve for diacetyl obtained from sterile, nonfat milk by the Owades and Jakovac method. Also shown for comparative purposes is the curve for diacetyl collected from distilled water, as well as the control curve (treated with color reagents directly). Data from which these curves were obtained are presented in Table 3, in order that the amount of variation normally encountered between duplicate tubes may be seen; at least 92 percent of diacetyl was recovered from sterile nonfat milk.

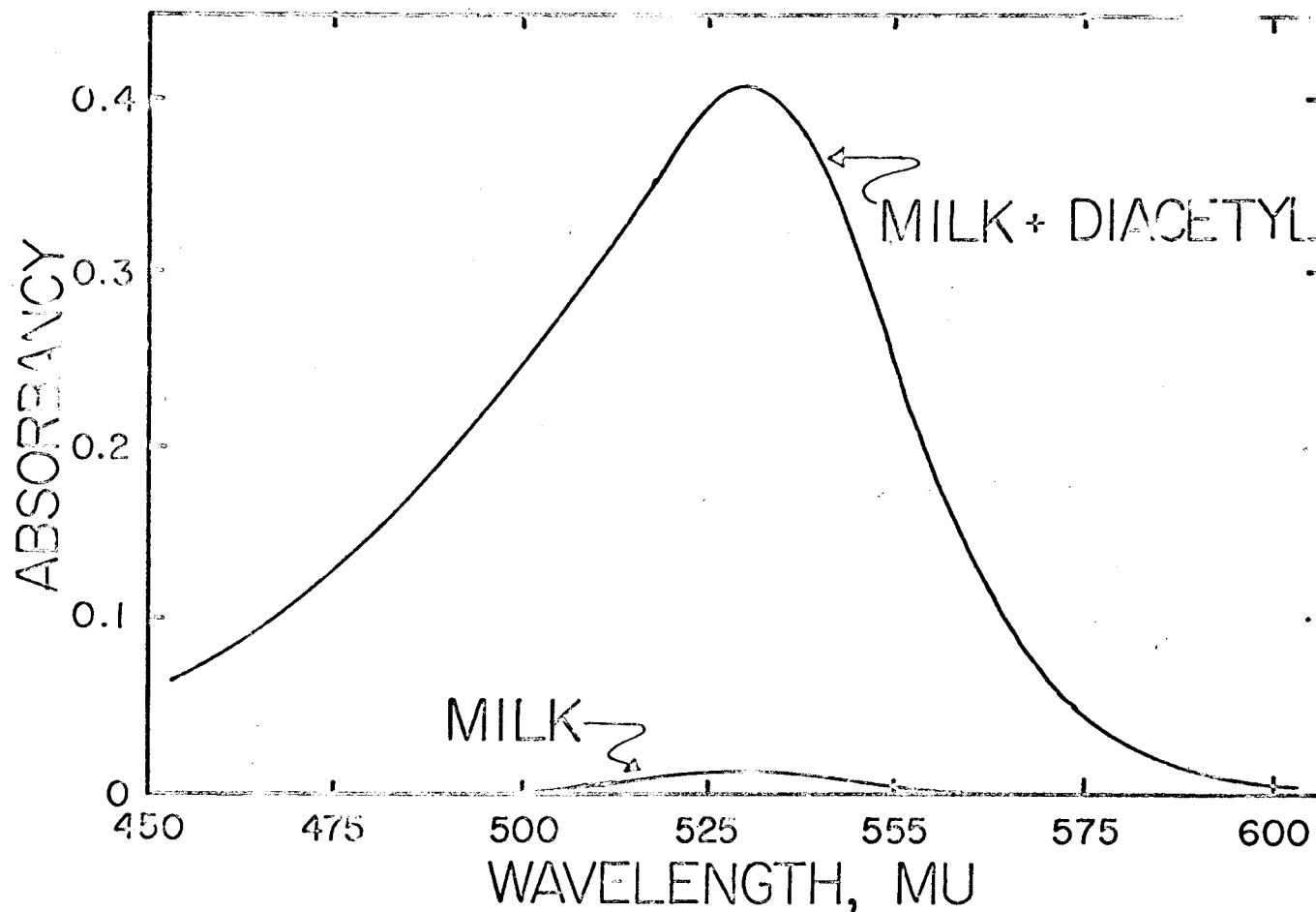


Figure 2. Absorption spectra (450 to 600 mμ) of diacetyl-hydroxylamine complex obtained from sterile milk with 2.0 ppm diacetyl (top curve) and from plain milk (lower curve) by the Owades and Jakovac method. Curves were obtained on a Cary No. 11 continuous recording spectrophotometer, using a reagent blank with plotting done on chart paper no. 1100.

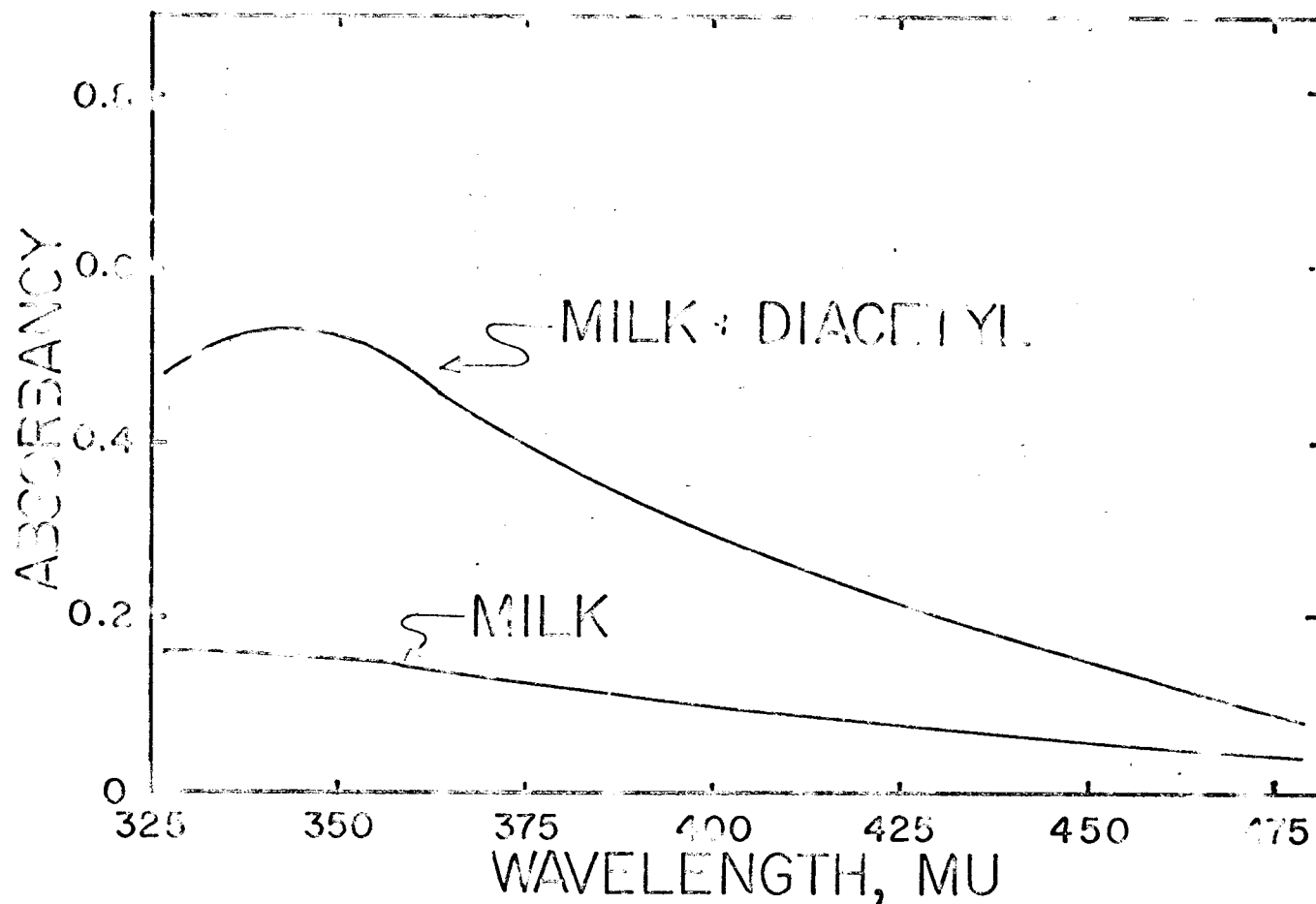


Figure 3. Absorption spectrum of diacetyl-3-3' diaminobenzidine· 4 HCl complex obtained from sterile milk with 2.0 ppm added diacetyl (top curve) and from plain milk (lower curve) by the method of Pien *et al.* Cary spectrophotometer was used as, in Figure 2, except employing hydrogen lamp for wavelengths below 375 mμ.

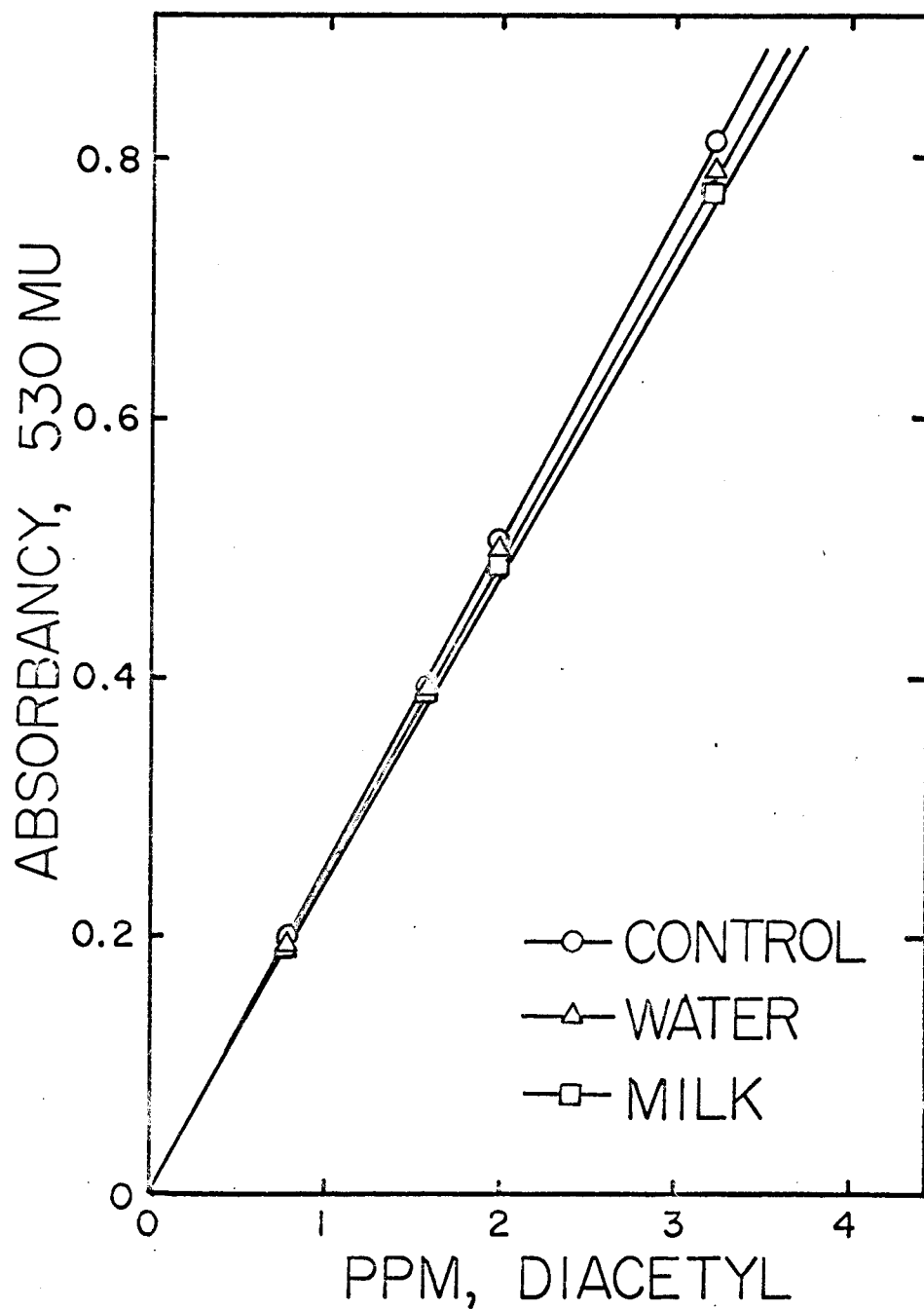


Figure 4. Absorbancy at 530 m μ of different amounts of diacetyl collected from water (triangles) and from milk (squares) by the Owades and Jakovac method. Control curve (circles) obtained from reading for different amounts of diacetyl added directly to buffered hydroxylamine and reacted with color reagents.

Table 3. Duplicate Absorbancy Readings at 530 m μ of Different Amounts of Diacetyl Collected from Water System and Milk System by the Owades and Jakovac Method*

System	Absorbancy at 530 mμ								% Recovery
	0.8 ppm		1.6 ppm		2.0 ppm		3.2 ppm		
	1	2	1	2	1	2	1	2	
Water	0.20	0.19	0.40	0.39	0.52	0.49	0.79	0.79	95 to 99
Milk	0.19	0.18	0.40	0.37	0.49	0.48	0.78	0.77	92 to 95
Control	0.21	0.20	0.42	0.41	0.53	0.50	0.82	0.81	100

* Control readings were obtained by adding color reagents directly (without sweeping by gas) to 1 ml of buffered hydroxylamine containing different amounts of diacetyl.

Effect of Different Gases

The effect of using different gases for sweeping diacetyl from a mixed-strain lactic starter culture is shown in Table 4. The use of air resulted in high values, perhaps due to chemical oxidative decarboxylation of alpha-acetolactic acid to diacetyl. There appeared to be no difference between nitrogen and CO₂ and tanks of the former were used, since they were less expensive.

Table 4. Effect of Different Sweeping Gases on the Amount of Diacetyl Found in a Mixed-strain Starter Culture Using the Method of Owades and Jakovac

Gas	Absorbancy at 530 m μ			Average ppm diacetyl
	1	2	3	
Air	0.64	0.62	0.62	2.7
N ₂	0.54	0.53	0.48	2.3
CO ₂	0.50	0.48	0.48	2.2

Effect of Antifoam, Acetoin, and 2, 3-Butanediol

To prevent undue foaming of milk cultures during analysis, it was necessary to use an antifoam agent (Foamkil; Nutritional Biochemical Corp., Cleveland, Ohio). Evaluation of the effect of this agent on absorbancy readings under different conditions is shown in Table 5. It may be seen that the use of antifoam had no influence on the results. It also can be seen from this table that acethymethylcarbinol (AMC) and 2, 3-butanediol (2, 3-BG) give essentially no color reaction with hydroxylamine and ferrous sulfate. Ethyl acetate and ethyl formate also gave no color.

Table 5. Effect of Antifoam on Absorbancy at 530 m μ of Diacetyl Collected from Different Systems and Reacted by the Owades and Jakovac Method

Analysis System	Absorbancy at 530 m μ			
	Minus Antifoam		Plus Antifoam	
	1	2	1	2
Water + 2.0 ppm diacetyl	0.45	0.45	0.45	0.45
Sterile milk	0.02	0.02	0.02	0.02
Sterile milk + 2.0 ppm diacetyl	0.48	0.47	0.45	0.45
Mixed-strain starter cultures	1.90	1.90	1.90	1.80
Water + 5.0 ppm AMC	0.005	0.001	--	--
Water + 5.0 ppm 2, 3-BG	0.005	0.000	--	--

Comparison Between Three Different Methods

Table 6 shows results comparing the different methods for diacetyl determination on single-and mixed-strain lactic streptococcus

starter cultures. From the table it may be seen that, in general, the A and B determinations made on the same cultures but on different days were in better agreement by the Owades and Jakovac and the Prill and Hammer method than by the method of Pien et al. Also, the latter method usually gave higher results than the two other methods; duplicate samples of the same culture tested by this method also were more variable.

Table 6. Diacetyl Produced (ppm) by Different Single- and Mixed-strain Lactic Streptococcus Starter Cultures as Determined by the Pien, Baisse, and Martin (PBM), Owades and Jakovac (O and J), and Prill and Hammer (P and H) Methods

Cultures	ppm Diacetyl found by 3 methods					
	PBM*		O and J		P and H	
	A [§]	B	A	B	A	B
<u>S. lactis</u> E	1.03	0.48	0.00	0.05	0.05	0.08
<u>S. lactis</u> C ₂	1.02	1.36	0.08	0.08	0.08	0.05
<u>S. cremoris</u> 1	0.55	0.36	0.03	0.05	0.05	0.05
<u>S. cremoris</u> C ₃	0.72	0.72	0.00	0.01	0.05	0.07
<u>S. diacetylactis</u> 26-2	3.28	1.52	1.84	1.76	--	--
<u>S. diacetylactis</u> 18-16	1.68	0.64	0.88	0.88	0.35	0.53
Mixed Culture E	0.72	0.30	1.84	2.36	0.54	0.42
Mixed Culture F	0.60	0.00	0.00	0.00	0.12	0.07
Mixed Culture G	0.36	0.48	1.28	0.96	0.55	0.58
Mixed Culture H	0.96	0.48	1.30	1.32	0.12	0.15

* As modified by Elliker (14).

§ A and B determination made in duplicate, but on different days.

Diacetyl Production and Destruction Patterns in Mixed-strain Starters

Typical Pattern

Figure 5 shows a typical pattern of diacetyl synthesis and destruction by commercial mixed-strain starter culture E. Diacetyl was synthesized up to about 5 ppm within 12 hours at 21° C followed by a rapid destruction down to less than 1 ppm in 36 hours by holding at the same temperature.

Cultural Variations

Figure 6 shows four different diacetyl curves by one single strain and three mixed-strain cultures. It may be seen that not all cultures behaved in an identical manner since some took longer to reach a peak than others and the rate of destruction in some cultures was slower than in others. The maximum production of diacetyl also was largely dependent upon the type of culture.

Temperature Effect

The formation and destruction of diacetyl was greatly influenced by the incubation temperature. The diacetyl curves observed during the manufacture of buttermilk on an experimental scale using two different mixed-strain starter cultures with known organisms at

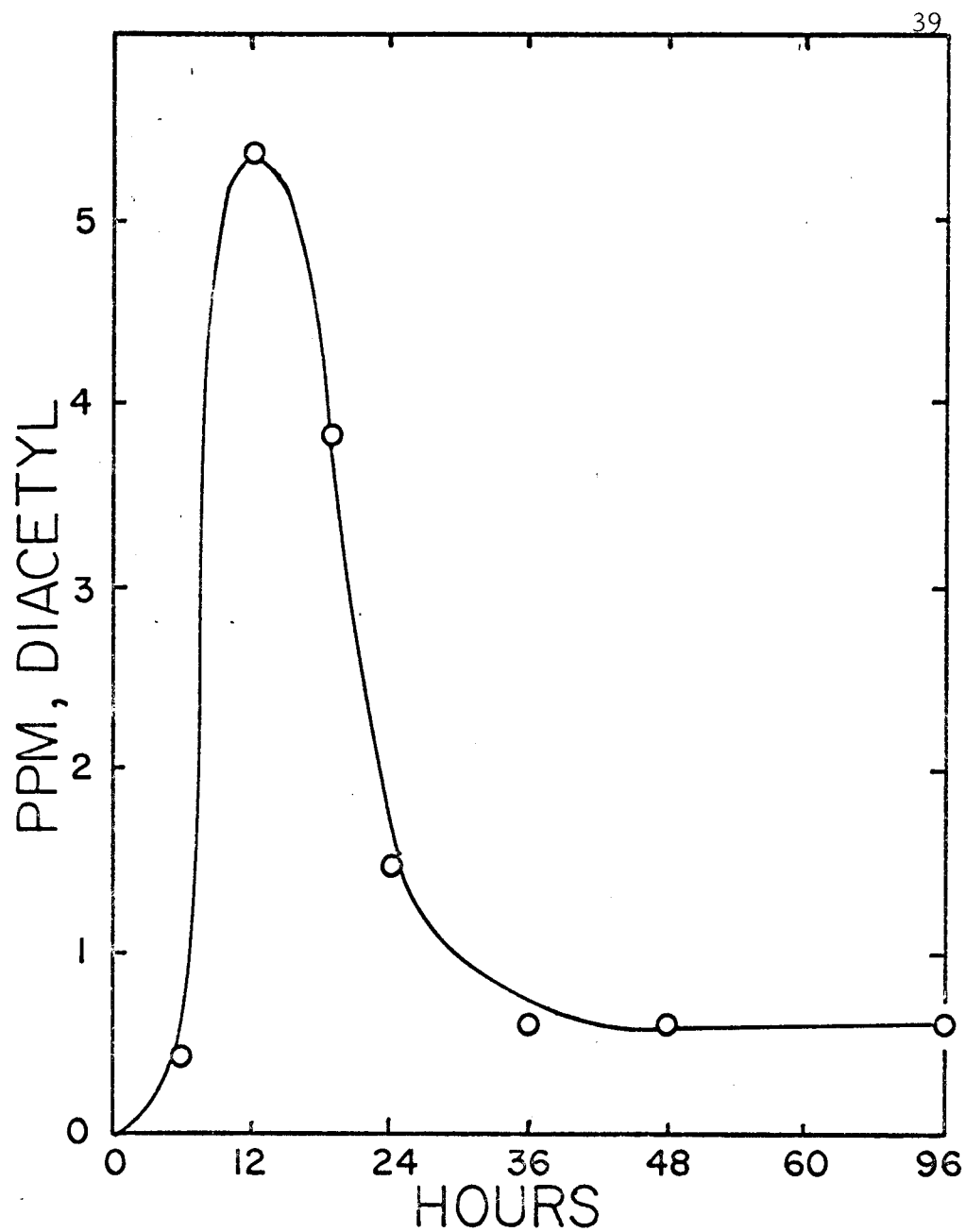


Figure 5. A typical diacetyl production and destruction pattern by mixed-strain starter culture E.

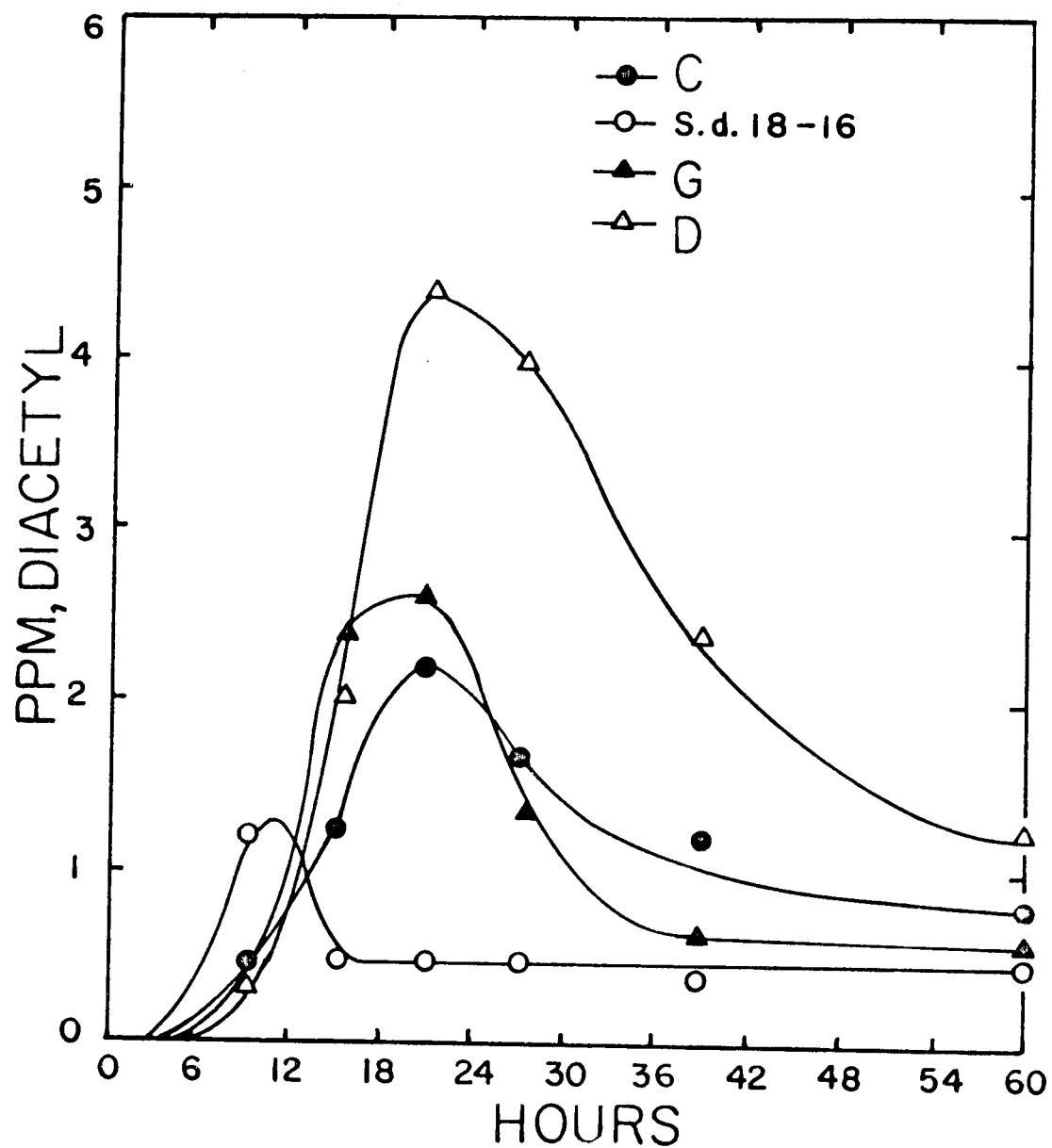


Figure 6. Diacetyl curves by one single strain and three mixed-strain starter cultures.

two different temperatures are shown in Figure 7. In both cultures, diacetyl formation started somewhat earlier at 32° C than at 21° C, however, diacetyl production at 21° C was about twice as much as at 32° C. Culture B, which contained S. diacetylactis strains, always showed greater production of diacetyl than culture A where Leuconostoc strains were the aroma producers.

Studies on Diacetyl Reductase

Relation between Enzyme Concentration and Reaction Rate

If a spectrophotometer cuvet contains DPNH and diacetyl reductase dissolved in buffer, the optical density at 340 mμ, due to DPNH, should remain constant until the substrate, diacetyl, is added to initiate oxidation of the cofactor. However, the crude enzyme showed a tendency to cause a decrease in the initial optical density even in the absence of diacetyl (Figure 8, curve with open circles). This was probably due to the presence of some other DPNH-oxidizing system (DPNH oxidase). As an alternative to purification, this activity could be eliminated by dilution (1:10) of the enzyme as shown in Figure 8 (curve with closed circles). Addition of substrate to the diluted enzyme (arrow) then resulted in rapid oxidation of the cofactor.

By plotting the time required for the 50 percent oxidation of

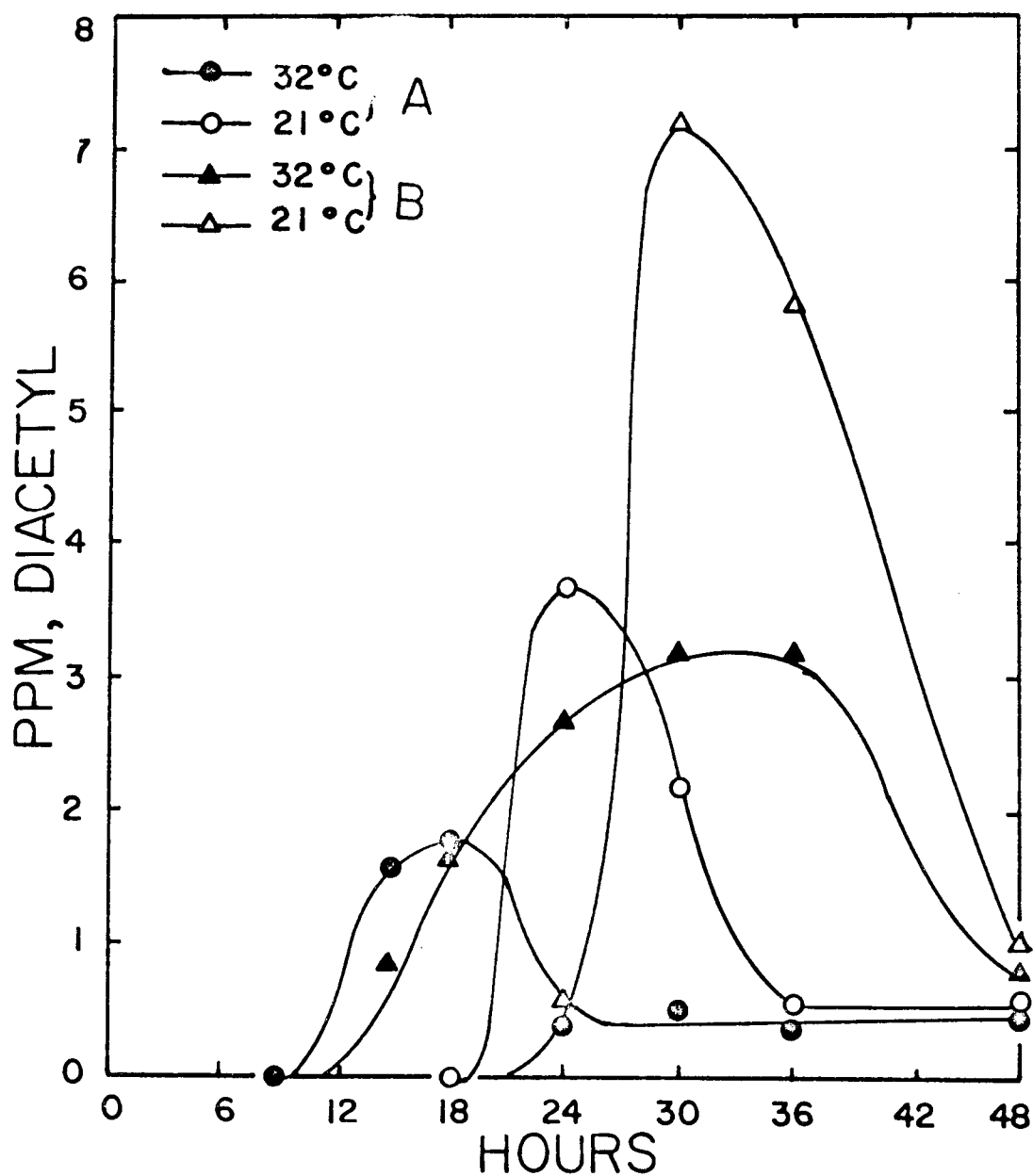


Figure 7. The effect of incubation temperature on diacetyl production and destruction by two different mixed-strain starter cultures. Culture A contained *S. cremoris* and *Leuconostoc* strains, and culture B consisted of *S. diacetylactis* strains in addition to *S. cremoris*.

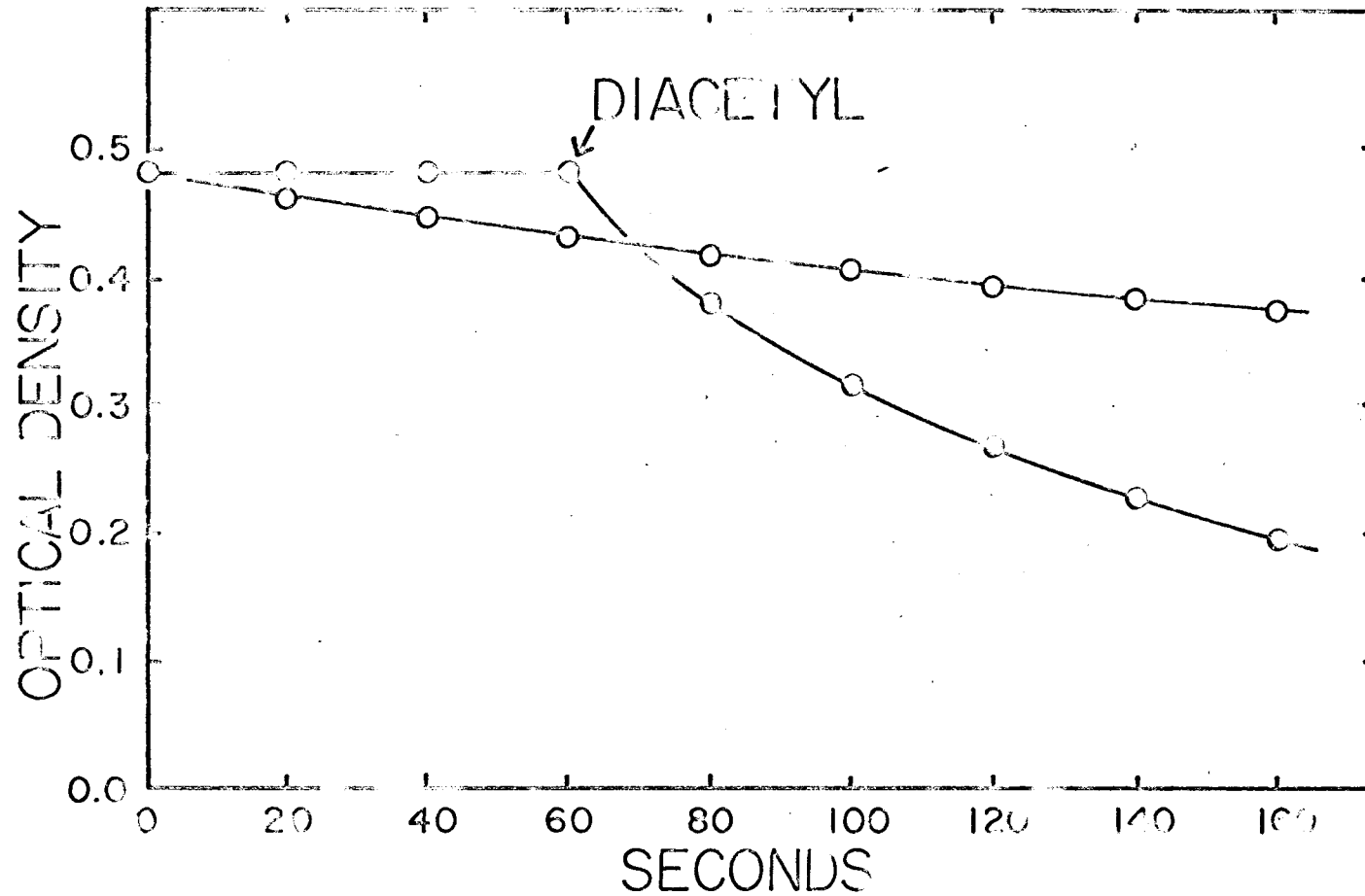


Figure 8. The effect of dilution of crude enzyme preparation on the assay system. The curve with open circles representing undiluted enzyme (50 mg/ml) showed a gradual oxidation of DPNH, whereas, the curve with closed circles (5 mg/ml enzyme) showed no decrease in optical density until diacetyl was added.

DPNH against enzyme concentration, activity of the enzyme could be observed as shown in Figure 9. A straight line was obtained when the reciprocal of the time was plotted against the concentration of enzyme. This facilitated enzyme assay and provided a convenient means of comparing the activity of different enzyme preparations.

Unit and Specific Activity of Enzyme

On the basis of the linear relation existing between enzyme concentration and the reciprocal of reaction time for a 50 percent reduction of the optical density of the cofactor, a system for enzyme unit determination was established. One unit was defined as the amount of enzyme which caused a 50 percent reduction in absorbancy when the reciprocal of time ($1/T$) was equal to 10^{-6} under standard assay condition. In other words, when one second was required for 50 percent reduction in absorbancy, one million units of enzyme would be present, or when one million seconds were required, one unit of enzyme would be present. The specific activity of diacetyl reductase was defined as units of the enzyme per mg of lyophilized crude enzyme solids.

Storage Temperature Effect

Figure 10 shows the stability of the enzyme stored at different temperatures. It may be seen that only short periods of storage of

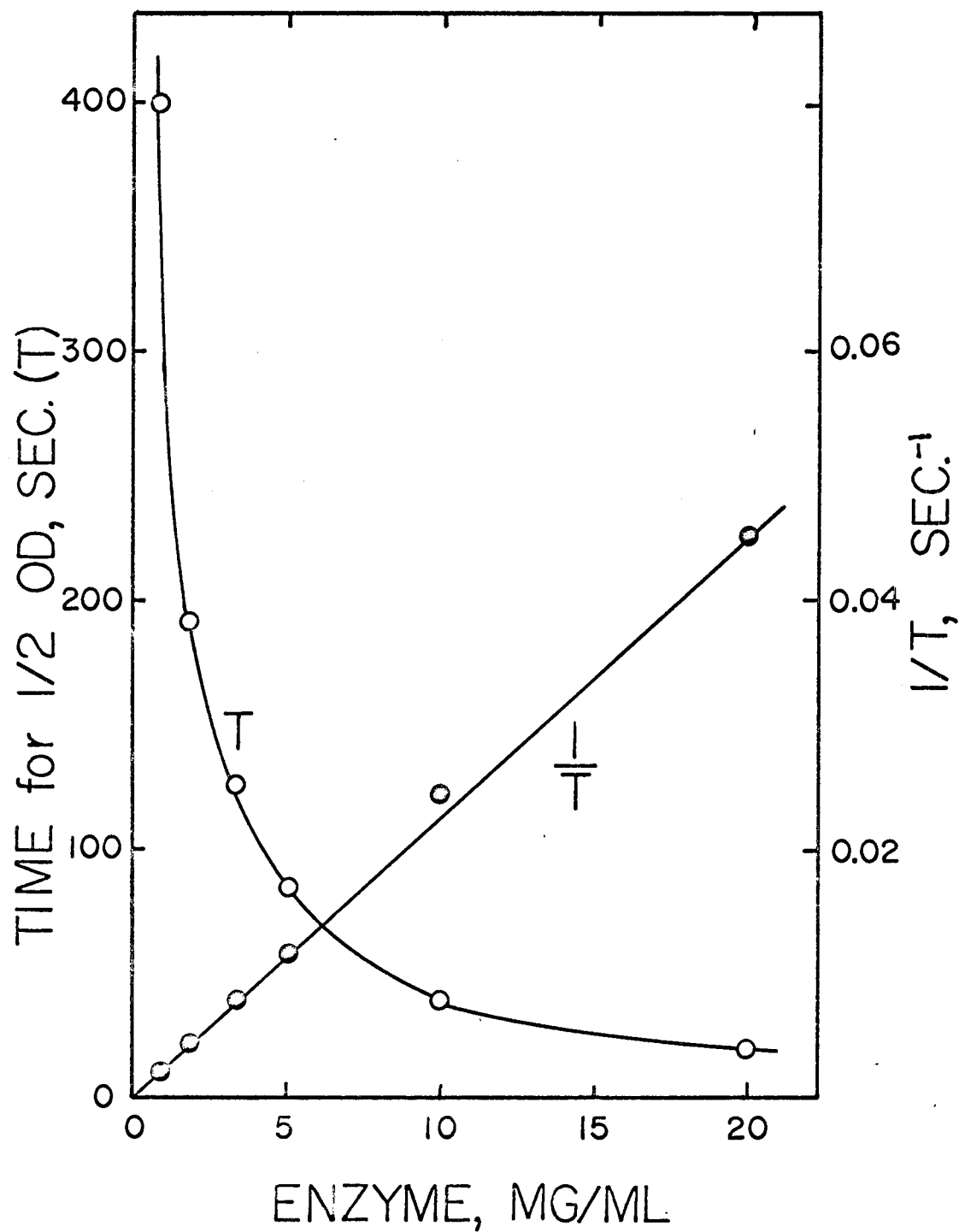


Figure 9. The relation between enzyme concentration and reaction time for oxidation of DPNH by diacetyl reductase in presence of diacetyl. See Table 1 for experimental conditions.

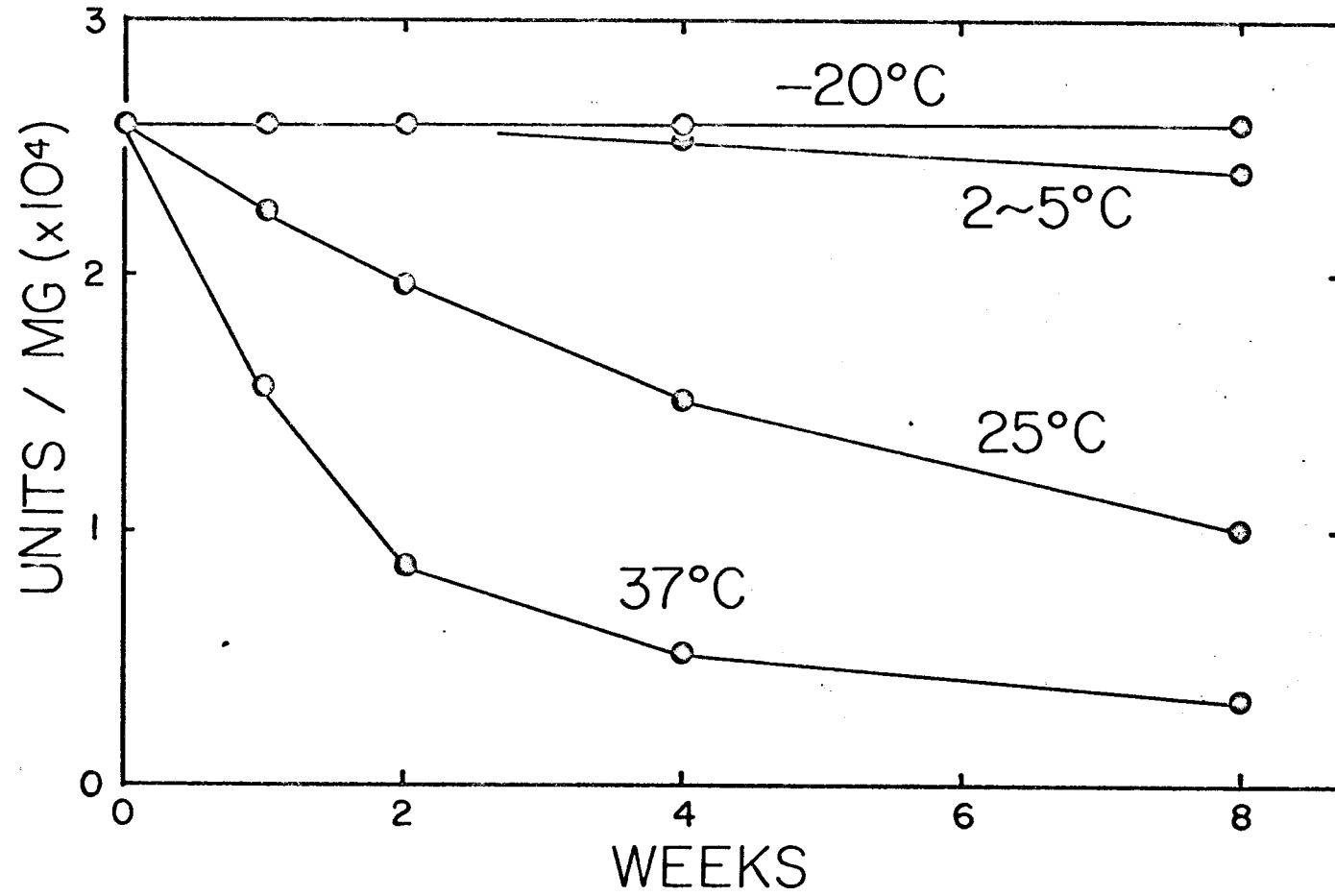


Figure 10. The effect of storage temperature on the stability of diacetyl reductase.

bulk quantities of lyophilized enzyme at room temperature would be desirable. Extended storage would require holding at 2 to 5°C and perhaps at - 20°C.

pH Effect

The effect of pH on the activity of diacetyl reductase and the stability of DPNH is shown in Figure 11. The optimum pH for the activity of the enzyme was between the pH values of 6 and 7. A rapid decrease of activity was found below pH 5.5, whereas DPNH was quite stable until pH 4.0, below which it was rapidly destroyed.

Effect of Cooling on Diacetyl Stability in Mixed-strain Starters and Dressed Cottage Cheese

Cooling Effect on Mixed-strain Starters

The rapid destruction of diacetyl produced in mixed-strain starter cultures was prevented by cooling the culture in a refrigerator (2 to 5°C) immediately after the maximum production was attained as shown in Figure 12. The two commercial mixed-strain cultures shown are placed in a refrigerator after incubation for 18 hours at 21°C; the diacetyl was stabilized at a relatively high level. Furthermore, some increase in diacetyl was observed under these conditions during storage.

This stabilizing effect, however, could not be observed when

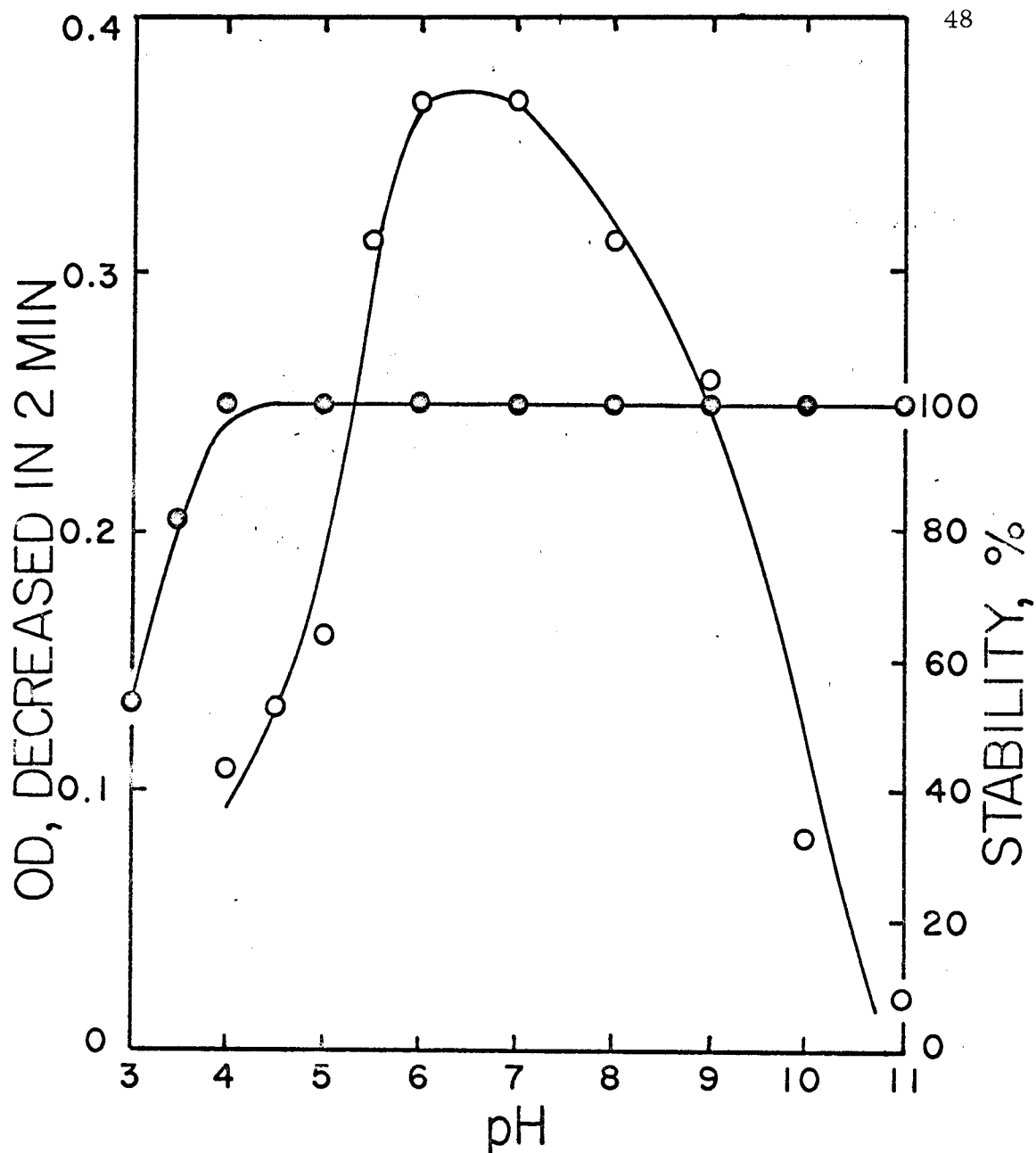


Figure 11. The effect of pH on the activity of diacetyl reductase (open circles) and the stability of DPNH (closed circles).

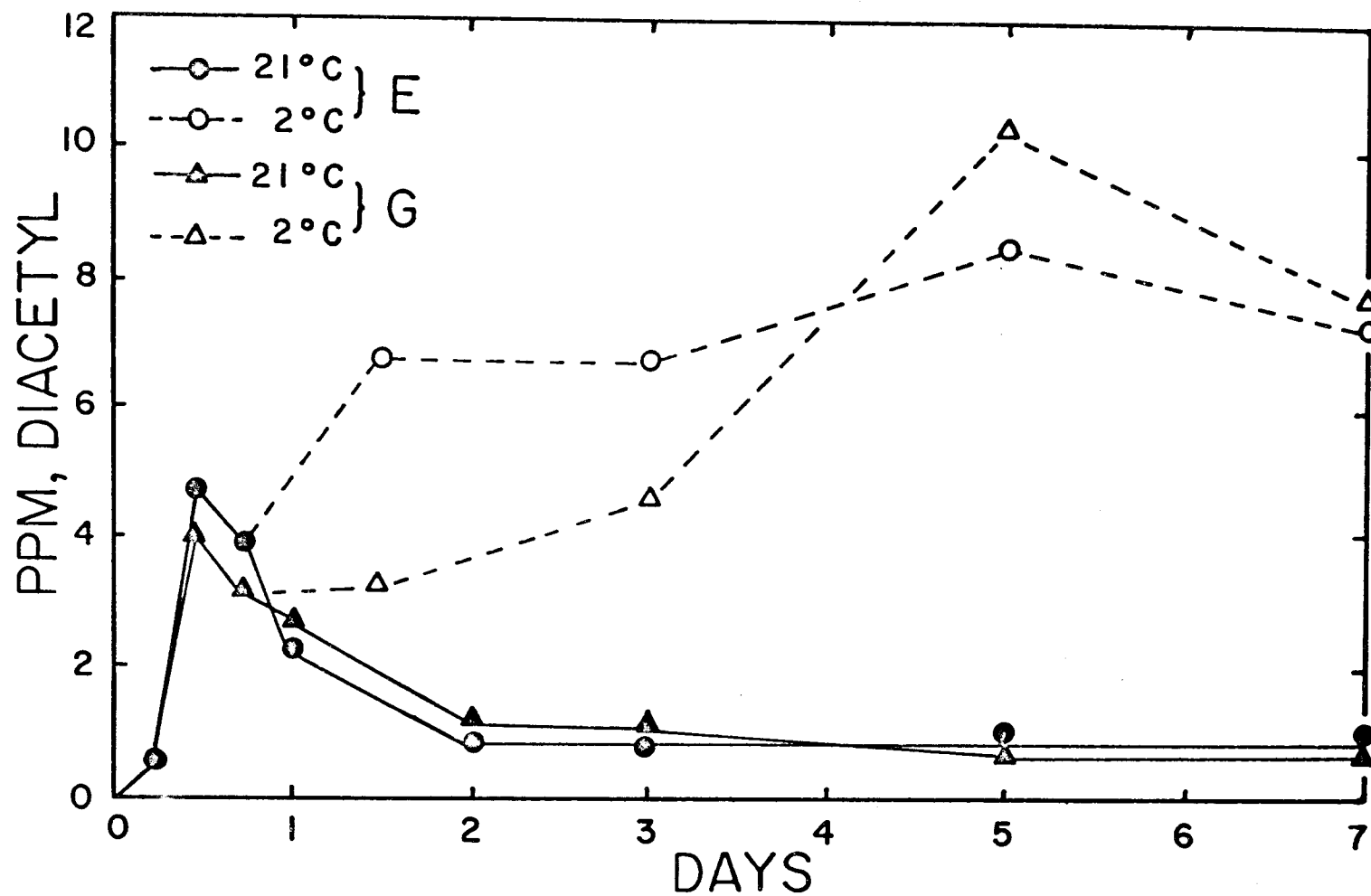


Figure 12. The effect of cooling on diacetyl stability in mixed-strain starter cultures. The broken lines show the diacetyl contents in bottles of 100 ml milk culture which were cooled promptly in a refrigerator (2° C) after 18 hours of incubation at 21° C, and the bottles for solid lines were continually held at 21° C.

the culture was cooled after the diacetyl was completely reduced to the minimum level. Cooling cultures before maximum diacetyl synthesis had occurred also resulted in an increase in flavor compound during storage. These results with culture E are shown in Figure 13.

Cooling Effect on Diacetyl in Dressed Cottage Cheese

A pronounced effect of cooling on diacetyl stability and enhancement was also seen in cottage cheese dressed with cultured cream. Diacetyl in cheese creamed with a 12-hour cream (12 percent fat) culture of S. diacetylactis 18-16 increased gradually in one month up to 7 ppm and 5 ppm when held at 5°C and 0°C, respectively; cheese dressed with non-cultured cream (control) showed only a trace amount (0.2 ppm) of diacetyl throughout the period (Figure 14).

Effect of Hydrogen Peroxide-Catalase Treatment on Diacetyl Stability in Single and Mixed-strain Starters

Effect on Mixed-strain Culture

A remarkable effect of the hydrogen peroxide-catalase treatment of pasteurized milk on the formation and stability of diacetyl in a mixed-strain starter culture can be seen in Figure 15. The 0.03 percent hydrogen peroxide without catalase inhibited the growth of the inoculated organisms completely, and catalase alone had no effect

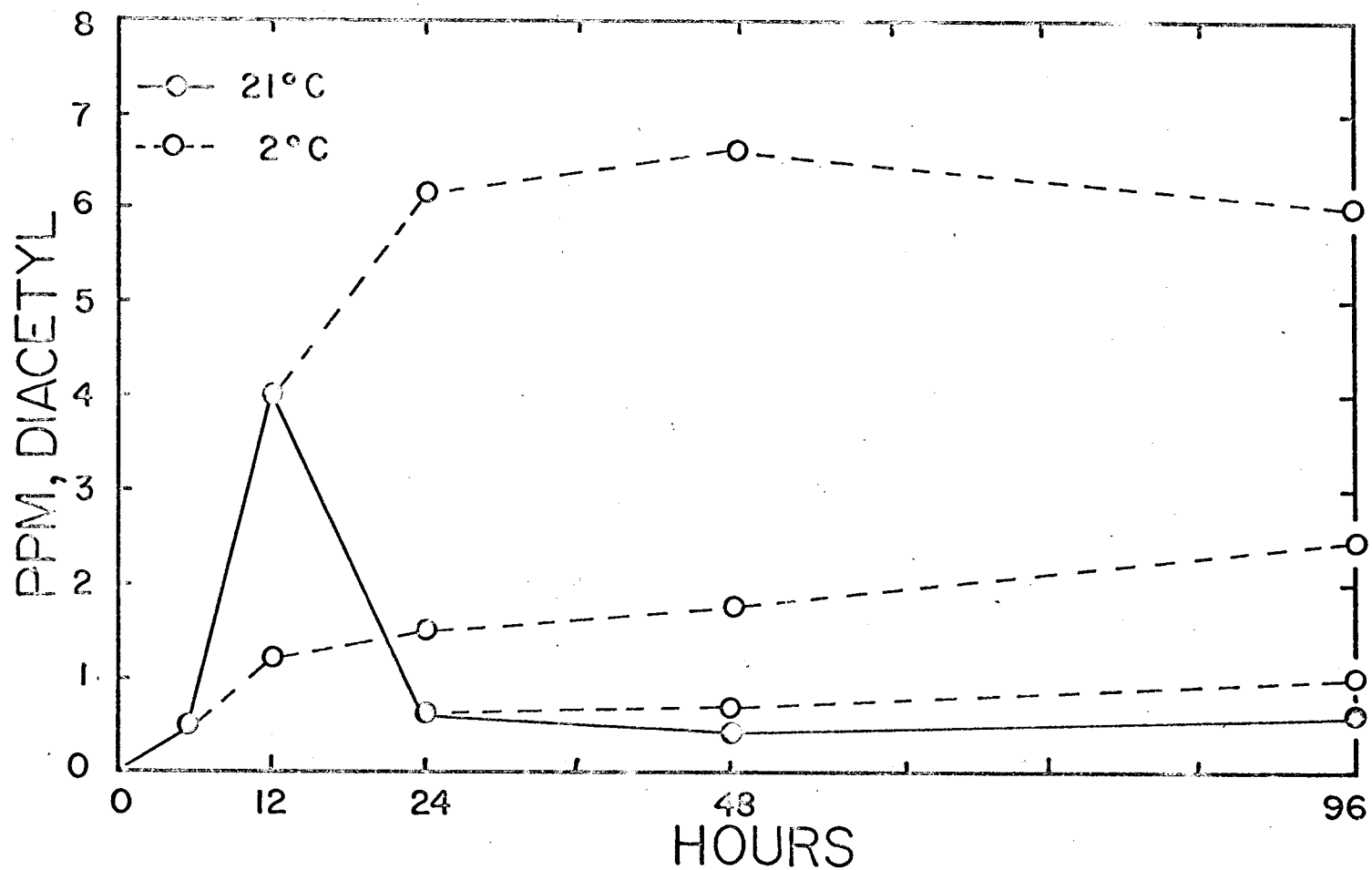


Figure 13. The influence of culture age on effectiveness of the cooling process in stabilizing diacetyl in mixed-strain starter culture E. Bottles were transferred into a refrigerator (2°C) after incubation periods of 6, 12, and 24 hours at 21°C.

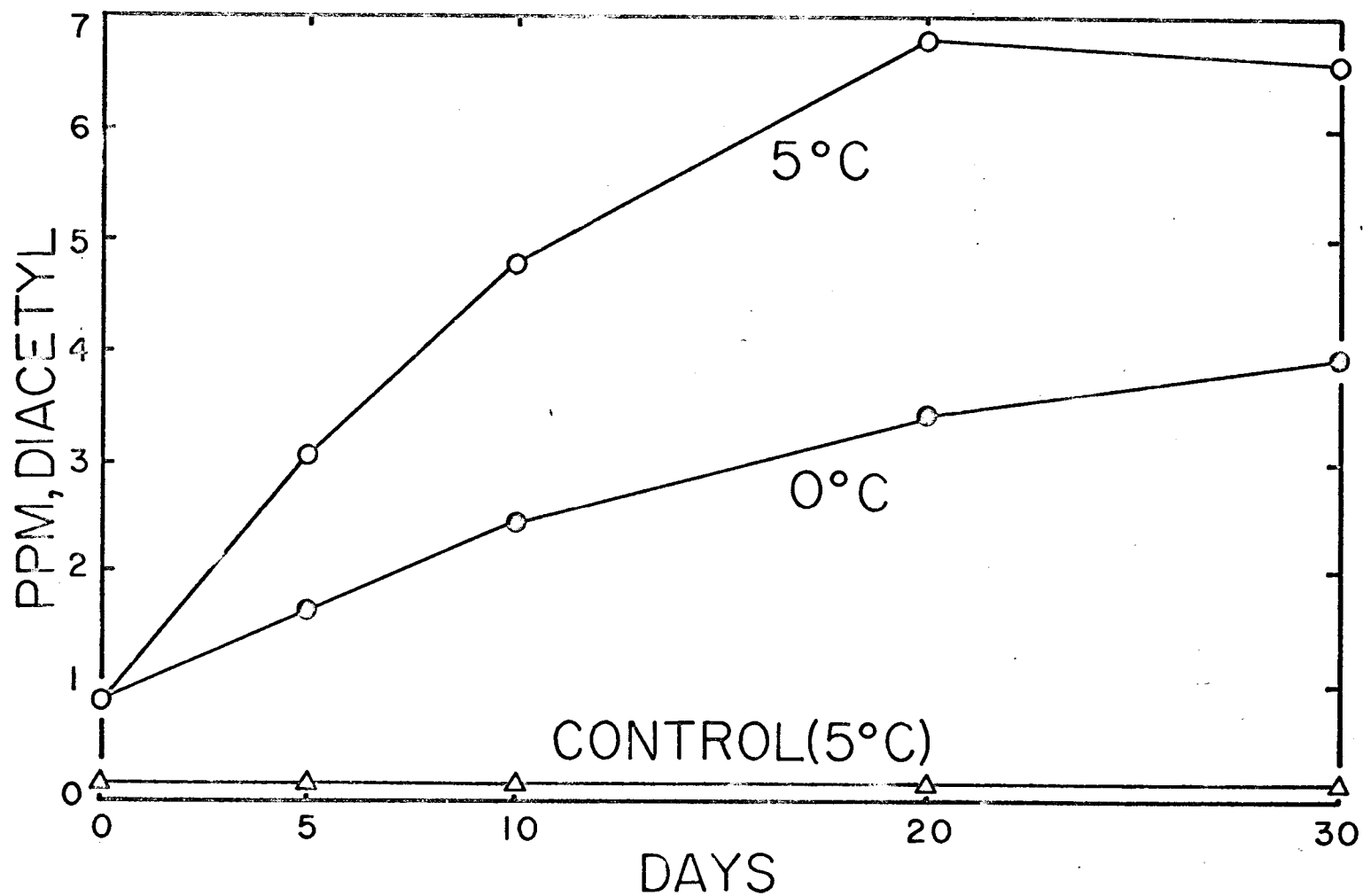


Figure 14. The effect of cooling on diacetyl enhancement in cottage cheese creamed with cultured dressing. The top (-O-) and middle (-●-) curves indicate diacetyl contents in cheese dressed with the cultured cream, and the bottom (-△-) curve is that for cheese dressed with non-cultured cream.

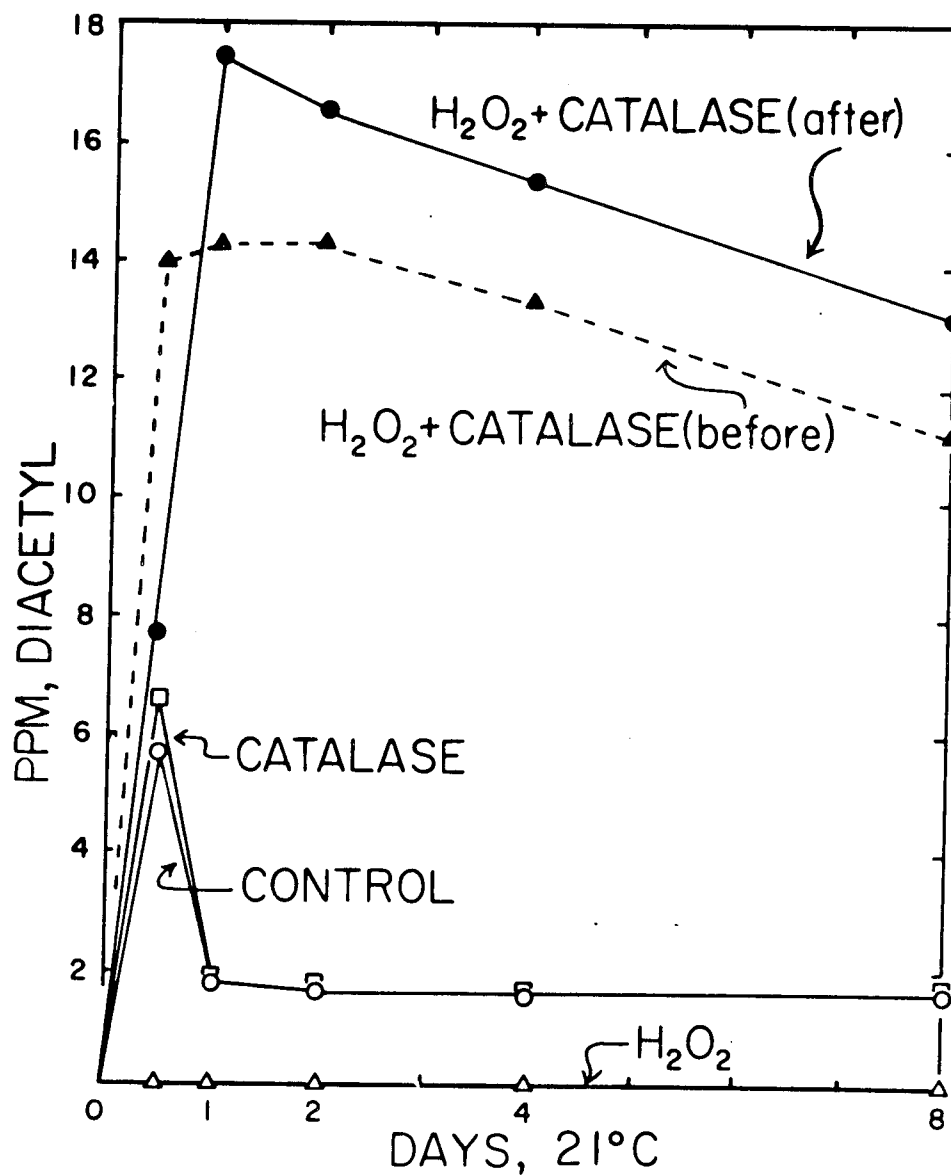


Figure 15. The effect of the hydrogen peroxide-catalase treatment on diacetyl stability in mixed-strain starter culture E. \circ : Sterile nonfat milk without treatment, \triangle : Milk treated with 0.03 percent hydrogen peroxide alone, \square : Milk treated with 0.004 percent catalase alone, \bullet and \blacktriangle : Milk treated with 0.03 percent of hydrogen peroxide followed by an addition of 0.004 percent of catalase. These treatments were performed before inoculation except one case (\bullet) which was treated after the inoculation.

on the diacetyl produced and destroyed. However, once the added hydrogen peroxide was decomposed in the milk by the action of the catalase, a tremendous increase of diacetyl occurred. Furthermore, the diacetyl was stable at 21°C for more than one week. Slightly higher diacetyl levels were found when the milk was treated after the inoculation but this also retarded the fermentation (acid production) significantly. Treatment before inoculation gave almost the same results and death of cells by exposure to oxidant was thus avoided.

Additional experiments were made to determine the effect of treating the milk at different times after inoculation on the level of diacetyl produced. These studies were made to gain information about the possible mechanism of the reaction as pointed out in the Discussion. Figure 16 shows that treatment with the hydrogen peroxide-catalase up to 12 hours had a stimulating effect. After this period, no significant increase in diacetyl could be seen, but the stabilizing effect of the treatment remained. The lower level of diacetyl at nine hours when the milk was treated immediately after the inoculation suggested that some inhibition of the inoculated organisms by the hydrogen peroxide-catalase treatment occurred. This was confirmed in later experiments the results of which are presented below.

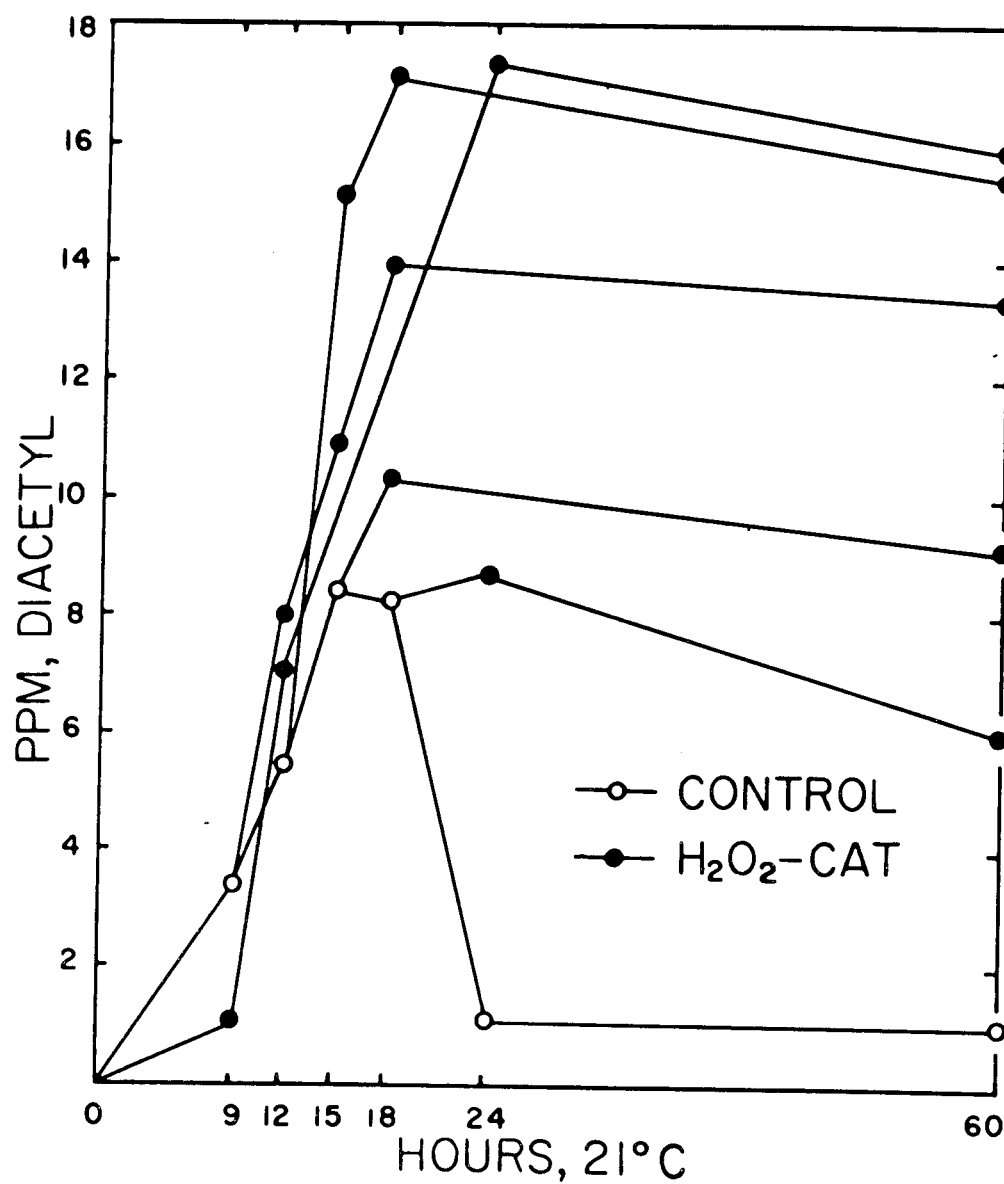


Figure 16. The effect of time of the hydrogen peroxide-catalase treatment on diacetyl production and stability in mixed-strain starter culture E. ○: Ten percent nonfat sterile milk without treatment, ●: Milk was treated with 0.03 percent of hydrogen peroxide followed by an addition of 0.004 percent of catalase after 0, 9, 12, 15, and 18 hours of incubation at 21°C.

Effect on Single-strain Culture

The same enhancing and stabilizing effect on diacetyl by the hydrogen peroxide-catalase treatment of pasteurized milk inoculated with a single strain of S. diacetylactis was found. For example, as shown in Figure 17, strain 18-16 produced only a low level of diacetyl when it was cultured in untreated milk. Treatment of the milk within nine hours after inoculation, however, enhanced the flavor level to as much as was achieved in the mixed-strain culture. It also was noted that the time beyond which the treatment was ineffective occurred earlier than in the case of the mixed-strain culture.

Effect of Different Concentrations of Hydrogen Peroxide-Catalase

The stability of diacetyl found when cultures were treated with 0.03 percent hydrogen peroxide and 0.004 percent catalase suggested that diacetyl levels might be regulated by adjusting the amount of oxidant used. This was found to be partially true. For example, Figure 18 shows the effect using a mixed-strain starter and two levels of hydrogen peroxide; it may be seen that less diacetyl was produced when the lower level of oxidant was used. Also, the diacetyl produced was somewhat less stable than the flavor compound elaborated in the presence of the greater concentration of hydrogen peroxide. Similar results were obtained with a single strain of

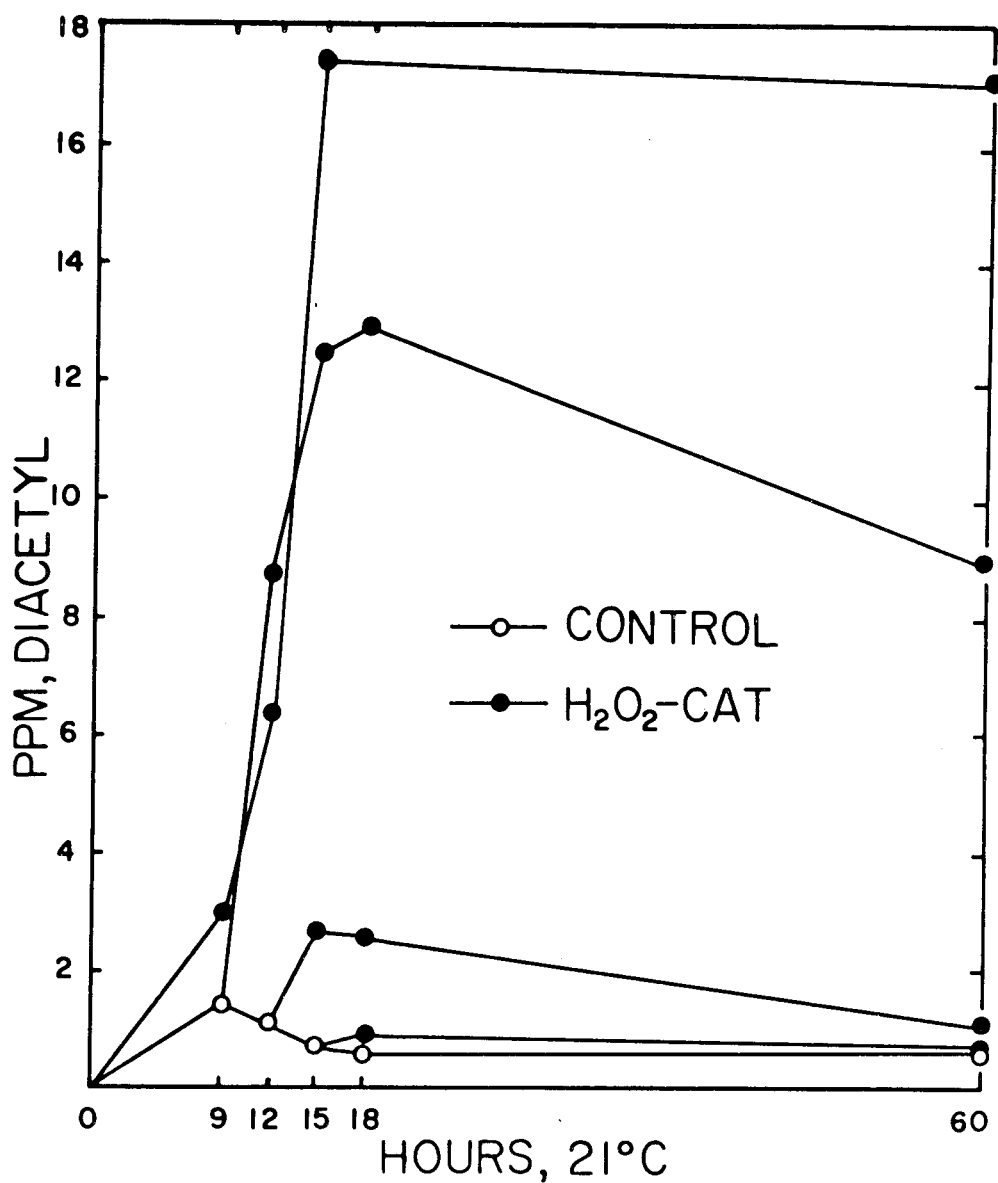


Figure 17. The effect of time of the hydrogen peroxide-catalase treatment on diacetyl production and stability in a single-strain culture of *S. diacetylactis* 18-16.
 ○: Ten percent nonfat sterile milk without treatment,
 ●: Milk was treated with 0.03 percent of hydrogen peroxide followed by an addition of 0.004 percent of catalase after 0, 9, 12, and 15 hours of incubation at 21° C.

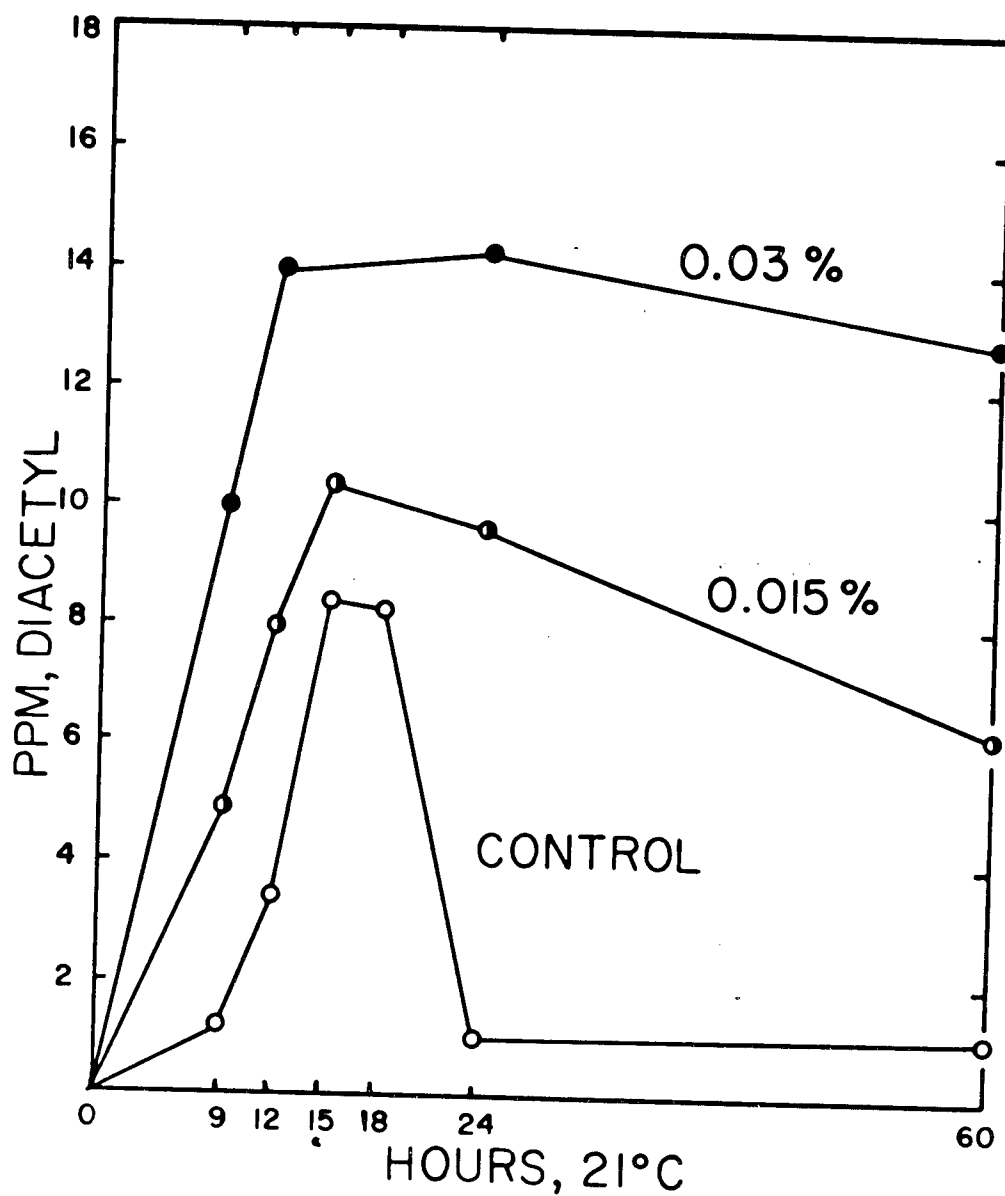


Figure 18. The effect of different concentrations of the hydrogen peroxide-catalase on diacetyl production by mixed-strain culture E. —○—: Ten percent nonfat milk without treatment, —●—: Milk treated with 0.03 percent of hydrogen peroxide followed by addition of 0.004 percent catalase, —○—: Milk treated with 0.015 percent of hydrogen peroxide followed by addition of 0.002 percent of catalase.

S. diacetylactis as shown in Figure 19.

Combined Effect of Hydrogen Peroxide-Catalase
Treatment and Cooling

Since the stabilization of diacetyl by the hydrogen peroxide-catalase treatment was reduced with the decrease in hydrogen peroxide concentration, a trial was made to measure the combined effects of the cooling process with this treatment on diacetyl production and stability. The results are shown in Table 7. Stabilization of diacetyl was definite even when the concentration of the hydrogen peroxide was low by the combination of these two processes. It again is evident from this table that the amount of diacetyl in dairy products may be easily controlled by regulating the hydrogen peroxide concentration for the treatment of the milk. It also may be noted from this table that the development of acid in the treated milk was somewhat retarded as the hydrogen peroxide concentration increased. The retardation, however, was not severe enough to interfere with normal plant routine, for example, in the manufacture of cultured buttermilk.

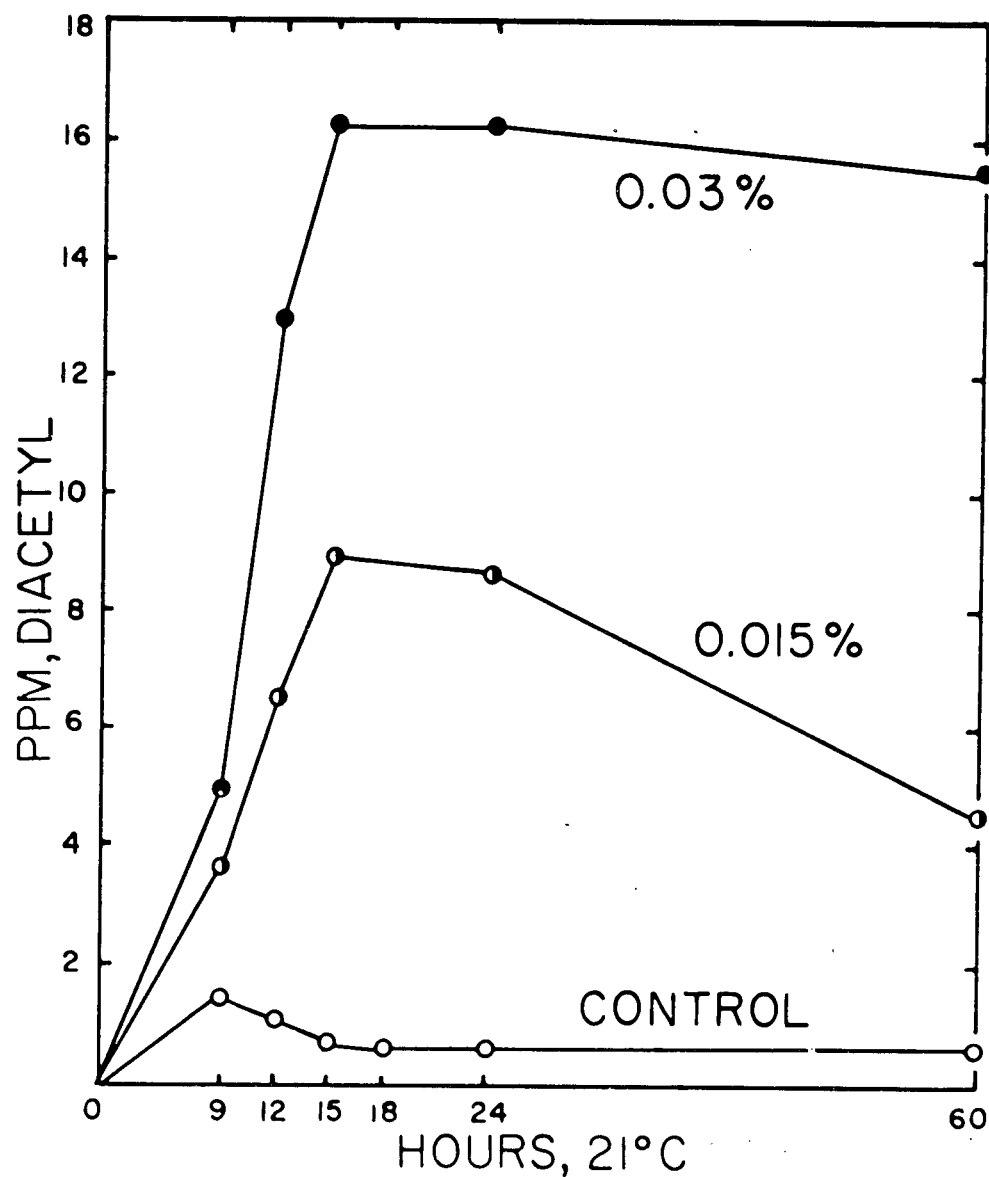


Figure 19. The effect of different concentrations of the hydrogen peroxide-catalase on diacetyl production by a single-strain culture of *S. diacetylactis* 18-16. —○—: Ten percent nonfat milk without treatment, —●—: Milk treated with 0.03 percent of hydrogen peroxide followed by an addition of 0.004 percent of catalase, —◐—: Milk treated with 0.015 percent of hydrogen peroxide followed by an addition of 0.002 percent of catalase.

Table 7. The Combined Effect of the Hydrogen Peroxide-Catalase Treatment and Cooling on Diacetyl Stability in Mixed-strain Starter Culture E. (The autoclaved milk was treated before inoculation and the diacetyl was determined from the bottles which received one percent inoculation.)

% H ₂ O ₂ Used	Hours for 0.85% acid at 21° C		ppm diacetyl after 5 days at	
	1%*	2%*	21° C	2° C
0.000	13.0	12.0	0.7	4.0
0.010	14.0	12.5	1.7	5.8
0.015	14.5	13.0	3.3	5.5
0.020	15.0	13.5	4.3	6.0
0.025	15.5	13.5	4.7	8.6
0.030	16.0	14.5	7.2	9.8

* Inoculum size

Enzymatic Removal of Diacetyl from Beer

Effect of Diacetyl Reductase and Pyridine Nucleotide

The first experiments on possible application of the crude diacetyl reductase enzyme from A. aerogenes to remove diacetyl from beer involved studies on the influence of different concentrations of enzyme and enzyme cofactor, reduced pyridine nucleotide (DPNH). Results of a typical experiment may be seen in Table 8. The added diacetyl was almost completely removed by holding for three hours at room temperature in presence of 20 mg of diacetyl reductase and 8 mg of DPNH. However, no effect was noted when these amounts

were reduced to 5 mg and 2 mg respectively.

Table 8. The Influence of Different Concentrations of Diacetyl Reductase and DPNH on Removal of Diacetyl from 20 ml of Beer After Inoculation for Three Hours at 25° C.

Initial	Enzyme [*] DPNH	Diacetyl in ppm		
		Final		
		5 mg 2 mg	10 mg 4 mg	20 mg 8 mg
0.50		0.45	0.30	0.10
0.35		0.35	0.25	0.08
0.25		0.25	0.18	0.05
0.10 ^{**}		0.10	0.10	0.05

* 35,000 units/mg

** Natural level of diacetyl in the beer sample; higher levels were obtained by diacetyl addition.

Effect of Coupling with Alcohol Dehydrogenase System

The amounts of diacetyl reductase and DPNH which were required for the destruction of added diacetyl in beer could be reduced by coupling the diacetyl reductase system to the alcohol dehydrogenase system as shown in Table 9. Only 2 mg of DPNH were sufficient to reduce the initial diacetyl level to less than one tenth the original concentration within 12 hours at 2° C, when 10 mg of diacetyl reductase and 16,000 units of alcohol dehydrogenase were also present. Although, enzymes appeared to regenerate DPNH, the levels of each

required seemed excessive for practical use.

Table 9. The Influence of Various Levels of Alcohol Dehydrogenase on Removal of Diacetyl from 20 ml of Beer by Action of Different Amounts of Diacetyl Reductase and DPNH During Incubation for 12 Hours at 2° C.

Additions to 20 ml beer*			
Diacetyl reductase (mg)**	DPNH (mg)	Alcohol dehydrogenase (units)	Final diacetyl (ppm)
0	0	0	3.7
1.0	0.1	4,000	3.5
10.0	1.0	4,000	1.8
10.0	1.0	8,000	2.0
10.0	2.0	16,000	0.35
10.0	2.0	32,000	0.35

*3.7 ppm added diacetyl

**35,000 units/mg

Table 10 presents data showing that the activity of diacetyl reductase was much greater in phosphate buffer at pH 7.0. Only 1 mg of enzyme and 1 mg of DPNH were necessary to remove 85 percent of added diacetyl in three hours at 25° C. The amount of DPNH required could be further reduced by coupling with alcohol dehydrogenase. These data indicated that the low activity of diacetyl reductase in beer was due to the low pH of the beverage which averaged 4.3. This was confirmed by the results in Table 11. By raising the original pH of 4.3 up to 5.0, the activity of diacetyl reductase in beer

was increased 20-fold. Furthermore, at pH 5.5, greater than 90 percent of the added diacetyl was destroyed within 12 hours at 2° C when alcohol dehydrogenase also was present. This table also suggests that the alcohol dehydrogenase was more sensitive to the low pH than the diacetyl reductase. For example, at pH 5.0 the 65 percent reduction in diacetyl catalyzed by diacetyl reductase could not be enhanced by addition of the DPNH regenerating enzyme. However, the alcohol dehydrogenase revealed a stimulatory effect at pH 5.5, where the coupled enzymes destroyed 93 percent of the diacetyl, but only 75 percent was removed by the principal enzyme alone.

Table 10. The Reduction of Diacetyl in Buffer by Diacetyl Reductase and by the Coupled Systems of Diacetyl Reductase and Alcohol Dehydrogenase

Additions to 20 ml of buffer*			
DPNH (mg)	Ethanol (ml)	Alcohol dehydrogenase (unit)	Diacetyl** reduction (%)
1.00	-	-	85
0.10	-	-	16
0.01	-	-	0
0.10	0.2	4000	70
0.01	0.2	4000	23

* 20 ml of 0.1 M potassium phosphate buffer at pH 7.0 with 2.6 ppm diacetyl and 1 mg of diacetyl reductase having 35,000 units/mg.

** Diacetyl was determined after holding three hours at 25° C.

Table 11. The Effect of pH on the Reduction of Diacetyl in Beer by Coupling Diacetyl Reductase and Alcohol Dehydrogenase.

pH of beer	Diacetyl reductase (mg)**	DPNH (mg)	Alcohol dehydrogenase (unit)	Diacetyl reduction (%)***
4.3*	10	2	-	3
	10	2	16,000	3
5.0	10	2	-	65
	10	2	16,000	65
5.5	10	2	-	75
	10	2	16,000	93
6.5	10	2	-	84
	10	2	16,000	90

* Unneutralized beer; higher pH values attained by addition of potassium hydroxide.

** 35,000 units/mg

*** Diacetyl determined after holding 12 hours at 2°C.

The amounts of the reaction components required for the removal of diacetyl in beer at pH 5.5 could be reduced by allowing a longer reaction time as shown in Table 12. Almost the same efficiency of flavor removal was obtained by half the amounts of diacetyl reductase, DPNH, and alcohol dehydrogenase by allowing 24 hours for incubation instead of three hours at 2°C.

Finally, the relative effectiveness of the components of the coupled systems in removing diacetyl was studied and typical results appear in Table 13. It may be seen that DPNH had the greatest

effect on diacetyl reduction, alcohol dehydrogenase had the least effect.

Table 12. The Effect of Reaction Time at 2°C on the Reduction of Diacetyl by Coupling Diacetyl Reductase and Alcohol Dehydrogenase in Beer in Which the pH was Adjusted to 5.5 with Potassium Hydroxide.

Beer adjusted to pH 5.5 (ml)	Diacetyl reductase (mg)**	DPNH (mg)	Alcohol dehydrogenase (units)	Diacetyl reduction (%)		
				(Hours at 2° C)		
				3	12	24
20	10	2	16,000	90	93	93
20	5	1	8,000	44	56	82

* Fortified with diacetyl to 3.4 ppm.

** 35,000 units/mg

Table 13. Effect of the Ratio between the Components of the Coupled Enzymes (diacetyl reductase and alcohol dehydrogenase) on the Reduction of Diacetyl in Beer (pH 5.5) Containing 3.4 ppm of Diacetyl.

Diacetyl reductase (5 mg)*	DPNH (1 mg)	Alcohol dehydrogenase (8000 units)	Diacetyl reduction (%)**
1	1	1	56
2	1	1	68
1	2	1	75
1	1	2	58
2	2	2	93
1	2	2	75
2	1	2	65
2	2	1	85

* 35,000 units/mg

** Diacetyl determined after 12 hours at 2° C.

DISCUSSION

Owades and Jakovac Method for Diacetyl Determination in Mixed-strain Starters

The advantage of the Owades and Jakovac method as applied to starter cultures in this study lies primarily in the fact that several samples can be analyzed simultaneously for diacetyl. This is a saving in time but, more important, this allows one to compare different cultures for flavor production without having to chill and hold samples until they are distilled. Such chilling practices may lead to inaccurate diacetyl values, since the diacetyl level still varies under this condition which will be described later. Also, the apparatus required is simple, inexpensive, and should make it possible for more dairies and culture supply houses to examine the production and stability of flavor and aroma in their single- and mixed-strain lactic streptococcus starter cultures.

Diacetyl analyses using the color formed as a result of the reaction with hydroxylamine to form dimethylglyoxime are more accurate than the determination based on reaction with diaminobenzidine. This may be seen from Figures 2 and 3; the dimethylglyoxime complex (Figure 2) has a fairly sharp absorption maximum at 530 m μ which is essentially absent in milk controls. The diaminobenzidine complex (Figure 3) shows broad absorption in the ultraviolet range,

with a maximum around 350 m μ ; milk controls, however, also absorb significantly in this region. Workers in the past, not having a spectrophotometer equipped with a UV light source, have used 425 m μ for reading this color absorbancy, and it may be seen from Figure 3 that such a practice would not be very satisfactory. Furthermore, to achieve an absorbancy reading of about 0.72 at 425 m μ , about 200 ppm of diacetyl is required. However, using the dimethylglyoxime end point, only about 3.0 ppm of diacetyl is required (Figure 4) for this absorbancy reading. Thus, the latter end point also provides much greater sensitivity in diacetyl determinations.

These advantages (accuracy and sensitivity) are reflected in Table 6; the Owades and Jakovac and Prill and Hammer methods were generally more reproducible, presumably due to the fact that they each employ the dimethylglyoxime end point. The accuracy of the Owades and Jakovac method as used in the present investigation also may be seen in Tables 3, 4, and 5, where repeated analyses show close agreement.

It is difficult to explain the higher diacetyl values found for the single and mixed cultures using the method of Pien et al. It is possible that carbonyl compounds other than diacetyl which react with diaminobenzidine are produced by some but not all lactic streptococcus organisms. Recent unpublished observations from these laboratories indicate that this may be the case. Also, since only 10 ml of

distillate are collected in the Prill and Hammer method, values obtained by this technique may be low. Consequently, a sample high in diacetyl may contribute to a high value in the next sample distilled, due to diacetyl accumulation in condensate in the distillation apparatus. But, since it was not the intent of the present work to study other diacetyl determination methods per se, no attempt was made to verify these possibilities.

Since it is known that S. lactis and S. cremoris organisms produce little or no diacetyl, it is felt that the results from the Owades and Jakovac method shown in Table 6 are accurate reflections of diacetyl production for the cultures listed. This method, therefore, is now being used routinely in this laboratory.

Diacetyl Production and Destruction Patterns in Mixed-strain Starters

Seitz et al. (61) reported that S. diacetylactis 18-16 synthesized and accumulated diacetyl in nonfat sterile milk incubated at 21°C. Build-up of the flavor compound lasted for about 24 hours and then it was decomposed rapidly. The authors concluded that this destruction of diacetyl was due to the action of diacetyl reductase which was substantiated by demonstration of the enzyme in the cell-free extracts of S. diacetylactis (62). Similar results were obtained in the present

study with mixed-strain starter cultures, with some differences in the diacetyl production-destruction curves being noted depending on the particular culture being examined. These differences were no doubt due to the presence of various strains making up the mixed cultures. For example, culture D shown in Figure 6 probably contained S. diacetylactis strains which dominated the culture, resulting in high diacetyl production and destruction. The other two mixed-strain cultures (C and G) may also have contained S. diacetylactis but they may have been suppressed in growth. Also, Leuconostoc may have been present in place of S. diacetylactis in these two cultures.

No direct evidence was obtained to indicate that the enzyme diacetyl reductase in these organisms was inducible. As a matter of fact, addition of diacetyl to milk subsequently inoculated with S. diacetylactis resulted in no change in the diacetyl production-destruction curve. Thus diacetyl present early in the fermentation did not cause induction of enzyme with resulting greater flavor loss. Levels of enzyme present in cells of S. diacetylactis grown under different conditions were not compared; however, glucose and citrate grown cells of A. aerogenes had equivalent diacetyl reductase activities.

The parabolic shape of the diacetyl curve must be due to the fact that diacetyl production exceeds the rate of destruction early in the fermentation; once diacetyl precursor (α - acetolactic acid), derived from a limited supply (0.2 percent) of milk citrate, is exhausted

no more diacetyl is produced but continuous destruction occurs resulting in a rapid decrease of the flavor compound.

The incubation temperature for mixed-strain starter cultures containing either Leuconostoc or S. diacetylactis as aroma bacteria was found to have a striking influence on diacetyl production and destruction (Figure 7). The optimum temperature for growth of Leuconostoc strains is between 20 and 25°C (3, p. 532-533), and that for S. diacetylactis is 26°C (37). Nevertheless, a longer lag period for diacetyl production occurred at 21°C than at 32°C. This probably was due to the beneficial effect of the higher temperature in reactivating the dormant organisms from the lyophilized state. Despite the longer lag in flavor production at 21°C, greater total diacetyl was produced by both types of cultures at this temperature. This may be explained by the greater activity of diacetyl reductase at the higher temperature which caused more destruction of diacetyl.

Figure 7 also emphasizes the importance of sampling time for quantitation of diacetyl in cultures. For instance, diacetyl production by culture B at 21°C was much lower than that at 32°C after 24 hours of incubation; however, the situation was just the opposite only six hours later.

Studies on Diacetyl Reductase

Experiments to partially characterize crude preparations of

diacetyl reductase were carried out in an effort to gain information which would be useful in eliminating the detrimental effect of the enzyme on culture flavor. Data useful in possible practical use of the enzyme in the brewing industry also were sought. A. aerogenes ATCC 8724 was selected as the enzyme source, since it had been reported to be a high producer of diacetyl reductase by Seitz et al. (61).

Many investigators have found that low pH conditions favor a better lactic culture flavor. Reports in the literature (10, 36, 38, 39, 55, 74) indicate that a pH level of about 4.3 is optimum for maximum diacetyl production by Leuconostoc organisms cultured in milk. Similarly, cultures of S. diacetylactis were found by Seitz et al. (60) to produce greater quantities of diacetyl when the pH was adjusted to 4.3 after logarithmic growth. The recent findings of Harvey and Collins (24), that a rapid increase of citrate uptake occurs in intact cells of S. diacetylactis and L. citrovorum below pH 6.0, explains the reason for this acid stimulation of diacetyl production. However, another benefit derived from the low pH is the retardation of diacetyl reductase under acid conditions. Even at pH 5.0, activity of the enzyme is drastically reduced and at pH 4.3 very little activity could remain (Figure 11).

The stability of lyophilized preparations of diacetyl reductase at 2 to 5°C and especially when frozen (Figure 10) indicates that the enzyme may be stored for eventual future use to possibly reduce the

level of diacetyl in fermented alcoholic beverages and citrus juices.

Effect of Cooling on Diacetyl Stability in Mixed-strain Starters and Dressed Cottage Cheese

The knowledge obtained about the pattern of diacetyl production and destruction during the fermentation of milk suggested possible ways to maintain diacetyl flavor at a desirable level in dairy products. The inactivation of diacetyl reductase is one possibility but commonly used enzyme inhibitors such as sodium azide, iodoacetic acid or parachloromercuribenzoate could not be used in food products. Acid inactivation of the enzyme might be effective, but this would not be compatible with body and other flavor properties desired in the finished product. Use of low temperatures to inhibit the enzyme is another possibility. Direct data regarding the effect of temperature on the enzyme are not available, but it is easy to suppose that a low temperature would retard the activity rate. W. K. Moseley of W. K. Moseley Laboratory, Indianapolis, Indiana first used cooled, young cultures of S. diacetylactis to improve the keeping quality of cottage cheese; enhanced flavor also was noted, probably because the high levels of diacetyl produced by the young cells was not destroyed at low temperatures. As was suspected, in the present study an obvious stabilization of diacetyl was obtained by cooling cultures before diacetyl was destroyed. The increase in diacetyl, also found at this

low temperature (2° C), may be explained on the basis of the oxygen dependent chemical conversion of α - acetolactic acid to diacetyl (12, 67). This chemical conversion was demonstrated by the increase in diacetyl at 0° C (Figure 14) where no bacterial activity would be expected. The failure of cooling to enhance diacetyl after maximum production would thus be due to the exhaustion of the precursor.

Effect of Hydrogen Peroxide-Catalase Treatment on Diacetyl Stability in Single- and Mixed-strain Starters

The hydrogen peroxide-catalase treatment of milk has been employed by the Cheddar cheese industry for the past several years in place of heat pasteurization of milk. The treatment also has been used successfully in the manufacture of Swiss cheese (43) and canned sterile whole milk (51), though heat was also used in the latter case. The purpose of this treatment is to destroy pathogenic bacteria normally present in raw milk as well as coliform and related bacteria.

The treatment as used in the present study has been applied for an entirely different purpose. The milk when treated with hydrogen peroxide-catalase after heat sterilization, was found to afford greater synthesis and stability of diacetyl when subsequently inoculated with commercial mixed-strain starter cultures or with S. diacetylactis. The stability of the diacetyl upon prolonged incubation of cultures at 21° C was striking and even greater stabilization was found when this

treatment was combined with the cooling process.

The effect of the hydrogen peroxide-catalase treatment on diacetyl enhancement apparently is due to the oxidative effect or effects of the oxygen liberated from the oxidant by action of the catalase. Considering the diacetyl synthesis pathway established by Seitz et al. (62), it appears that there may be three possible sites of action for the oxygen liberated to act in stabilizing flavor. Figure 20 shows these possibilities. Any correct explanation has to deal with both enhanced synthesis and stability and each are considered in the figure; for example, I and II are concerned more with synthesis while III is concerned with stability. Let us consider each of these separately.

I. Direct Effect of Oxygen on α -acetolactic Acid.

There are several reports which support this first possibility. Pette (52) found that oxygen was effective in stimulating diacetyl formation mainly during the period of vigorous fermentation of citric acid by mixed-strain starter cultures. He postulated that an intermediate substance formed from citric acid was oxidized to diacetyl. De Man (12) reported that α - acetolactic acid which accumulated during the fermentation of pyruvic acid by B. cremoris, was transformed into diacetyl by decarboxylation on heating in the presence of air. He also found α -acetolactate decarboxylase in cells of B. cremoris, and postulated that in mixed-strain starters, souring cream

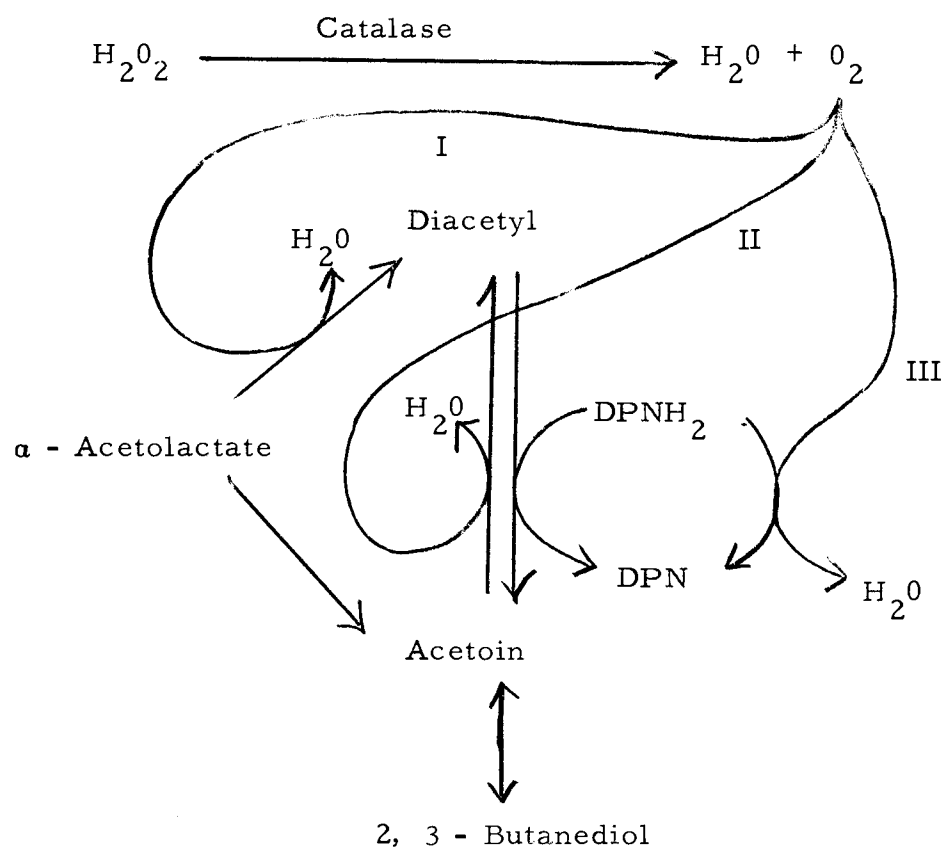


Figure 20. Three possible mechanisms of action for hydrogen peroxide-catalase enhancement of diacetyl production and stability in single or mixed-strain lactic starter cultures.

and butter, α - acetolactic acid was excreted by the aroma bacteria and then oxidatively decarboxylated to diacetyl in the presence of oxygen. Täufel and Behnke (67) demonstrated a conversion of synthetic α - acetolactic acid to both diacetyl and acetoin by cell-free extracts of aroma bacteria in the presence of air.

II. Oxidative Effect on Acetoin

Data from this study and from the work of Seitz (62) have clearly established that diacetyl is irreversibly converted to acetoin by the action of diacetyl reductase in mixed-strain starter cultures under normal conditions. However, this situation could be altered if an extraordinary high concentration of oxygen, such as in milk treated with hydrogen peroxide-catalase, was present. A possibility for the oxidation of acetoin back to diacetyl must be considered under these conditions. Furthermore, there are some reports by investigators who have considered acetoin to be the precursor of diacetyl. Nikkila (44) reported that the production of acetoin by malt-aroma bacteria (S. lactis var. Maltigenes) and S. paracitrovorus preceded diacetyl and 2, 3-butanediol formation. Kandler (30) believed that α - acetolactic acid was decarboxylated to acetoin which subsequently was oxidized to diacetyl and reduced to 2, 3-butanediol.

One experiment was carried out to test the possibility that acetoin could be oxidized to diacetyl by hydrogen peroxide-catalase.

Data were not presented in the results section because more experimentation in this area is needed. Briefly, however, the action of oxidant and enzyme on acetoin added to a culture filtrate of a mixed-strain starter resulted in a negligible increase in diacetyl (0.3 ppm).

III. Effect on DPN/DPNH Ratio

The last possibility was based on an assumed competition between diacetyl and oxygen for the hydrogen from DPNH. If oxygen from the hydrogen peroxide could oxidize DPNH more easily than diacetyl, then the DPN/DPNH ratio would increase and diacetyl reductase would be unoperative, resulting in stabilization of diacetyl in the product. An experiment was carried out in buffer to test this possibility, but the hydrogen peroxide-catalase had no effect on DPNH alone. However, in the presence of crude diacetyl reductase enzyme preparation from A. aerogenes, hydrogen peroxide-catalase stimulated the oxidation of DPNH. This suggested that other enzymes present in the extract which required reduced cofactor were stimulated by the treatment.

Enzymatic Removal of Diacetyl from Beer

Diacetyl could be removed when beer was treated with high concentrations of diacetyl reductase and DPNH (Table 8). The amount of enzyme needed could be reduced by allowing longer reaction

times but substrate quantities of DPNH were still required, since the reduced coenzyme serves as the hydrogen donor for diacetyl reduction. Use of purified cofactor in beer to control diacetyl off-flavor would be too expensive even if application of the enzyme was successful from other standpoints. Therefore, the possibility of coupling diacetyl reductase and alcohol dehydrogenase to regenerate DPNH was considered as shown in Figure 21. Support for the possible use of the coupled system was obtained from results of preliminary experiments shown in Figure 22, where the regeneration of DPNH was observed after the addition of alcohol dehydrogenase and substrate.

Trials with the coupled systems also succeeded in beer where DPNH was regenerated by the action of alcohol dehydrogenase. In this case, the alcohol in beer served as the hydrogen source. The efficiency of the coupled system in beer, however, was low (Table 9). There is no doubt that this low activity was at least partly due to the low pH of beer, since diacetyl was readily decomposed when the pH level in beer was raised up to 5.5. Furthermore, pH studies (Figure 11) showed that the activity of this enzyme was greatly inhibited around pH 4.5. Thus, further studies are required to find means of overcoming this limiting factor before application of diacetyl reductase in the brewing industry can be made.

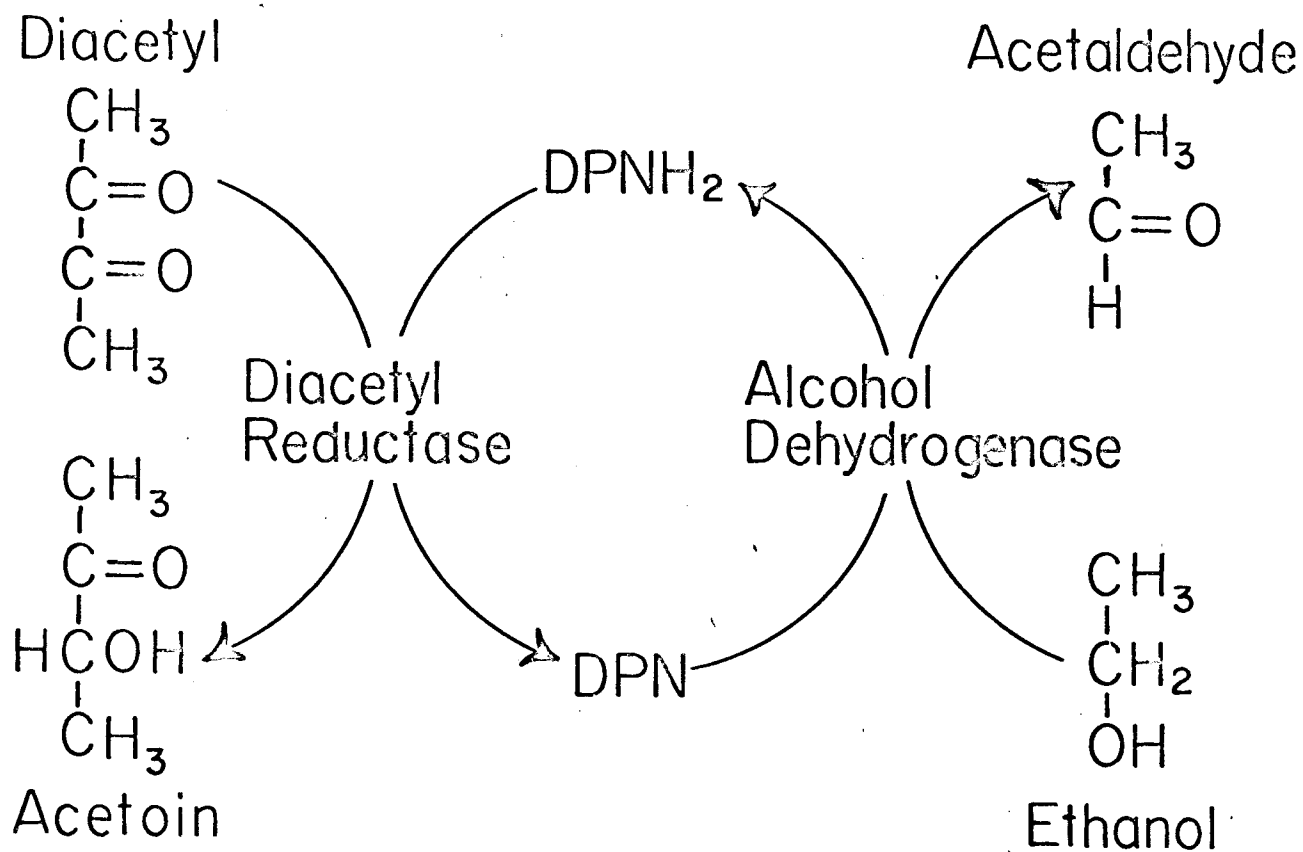


Figure 21. A schematic pathway for regeneration of reduced pyridine nucleotide (DPNH) via coupling to alcohol dehydrogenase.

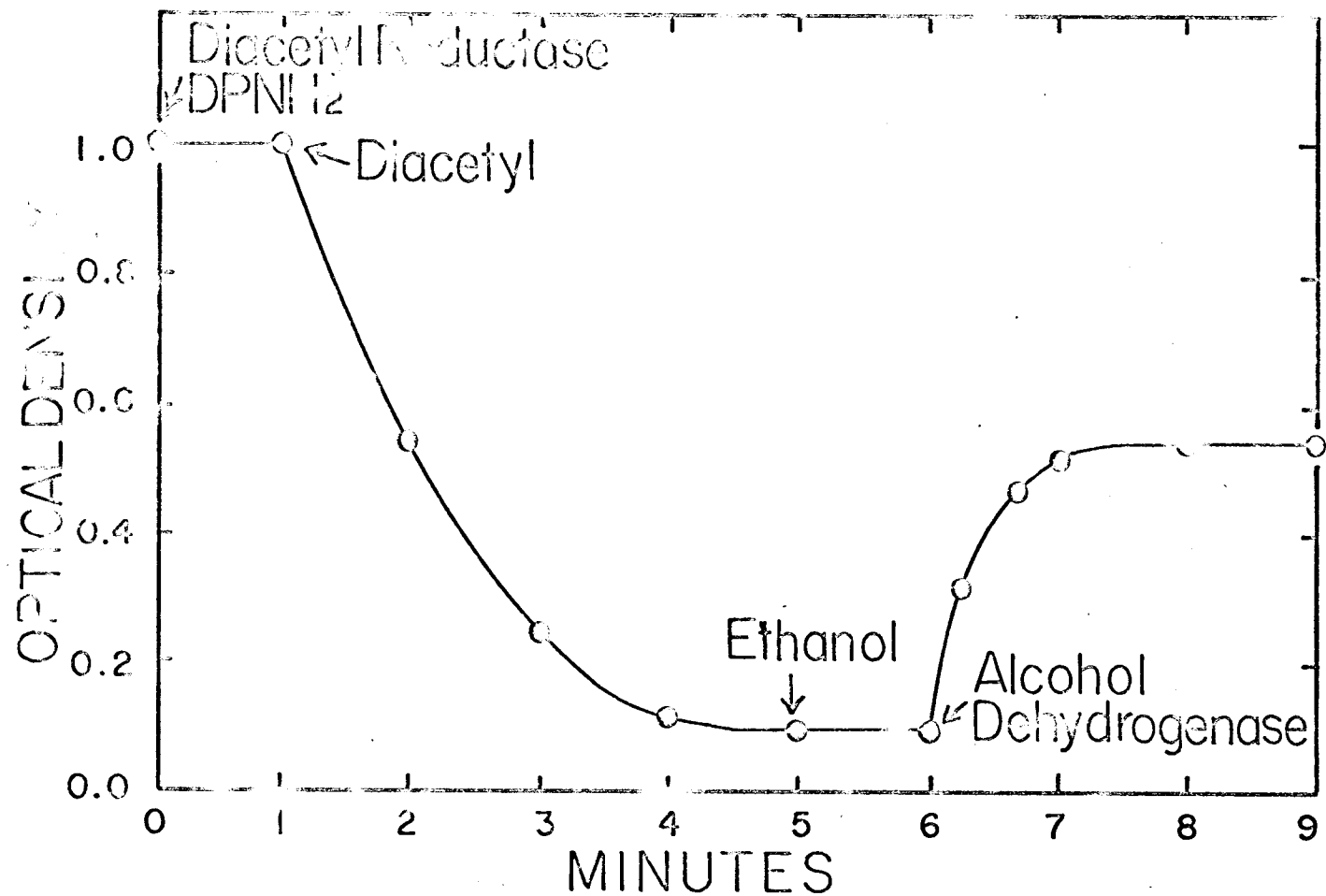


Figure 22. Regeneration of reduced pyridine nucleotide (DPNH) via coupling diacetyl reductase to alcohol dehydrogenase. The amounts of components in the reaction mixture were 0.5 mg of diacetyl reductase (17,500 units), 0.4 mg of DPNH, 86 μ g of diacetyl, 16,000 units of alcohol dehydrogenase, 0.1 ml of 95 percent ethanol, and sufficient of 0.1 M potassium phosphate buffer to provide a final volume of 3.0 ml.

SUMMARY

This investigation on flavor (diacetyl) control in dairy products and beer was carried out in an effort to stabilize diacetyl in dairy products and to remove diacetyl from beer.

A method recently described for the determination of diacetyl in beer was modified to use for analysis of flavor in dairy products. Twelve samples were assayed simultaneously, using a gassing manifold. Twenty-milliliter samples immersed in 65°C water bath were purged with nitrogen gas and the emerging diacetyl trapped as dimethylglyoxime in buffered hydroxylamine and converted to a colored complex by reaction with ferrous sulfate. Advantages of the method over two other procedures with which it was compared included greater accuracy and sensitivity and use of simple, inexpensive equipment. Chief advantage is that the procedure allows a number of samples to be tested at the same time, which permits accurate comparative data on diacetyl production by lactic streptococcus starter cultures to be obtained.

The patterns of diacetyl production and destruction during the fermentation of milk by single or mixed-strain starter organisms varied with the types of organisms as well as with the incubation temperatures. In a commercial mixed-strain containing S. diacetylactis and Leuconostoc as aroma bacteria, the diacetyl

increased up to 5 ppm within 12 hours, and then decreased rapidly to around 2 ppm over the next 12 hours. In some other cultures, the maximum diacetyl production came somewhat later and decomposition occurred more slowly. Diacetyl production by rejuvenated lyophilized mixed-strain cultures was found to begin sooner at 32°C than at 21°C, but the amount of flavor produced at 32°C was lower than at 21°C.

The enzyme diacetyl reductase obtained from cells of A. aerogenes showed an optimum activity over the pH range from 6.0 to 7.0; its activity was greatly inhibited below pH 5.0 and above pH 10.0. The coenzyme DPNH was quite stable until pH values below 4.0 were encountered. The crude preparation of diacetyl reductase could be stored at - 20°C for eight weeks without loss of activity.

Destruction of diacetyl during milk fermentation at 21°C could be prevented by cooling the cultures down to 2°C immediately after the maximum production of diacetyl. More than 7 ppm of diacetyl could be achieved in two different commercial mixed-strain starter cultures which were transferred to a refrigerator after 18 hours of incubation at 21°C, and stored for seven days; diacetyl levels in the same two cultures held at 21°C continuously were below 1 ppm. Stabilization by cooling was not effective when cultures were transferred to a low temperature after diacetyl had decreased to the minimum level. The cooling effect was also useful in enhancing

diacetyl in cottage cheese dressed with cream in which S. diacetylac-
tis had grown for 6 to 12 hours. The diacetyl level in such cheese
increased up to at least 7 ppm during 20 days of storage at 5°C.

An outstanding effect on both synthesis and stability of diacetyl
produced during the fermentation of milk by single or mixed-strain
starter cultures was found by treating the milk with hydrogen perox-
ide-catalase. When the heat sterilized milk (121°C for 13 minutes)
was treated with 0.03 % hydrogen peroxide and 0.004% catalase
before inoculation, diacetyl production reached 14 ppm and only de-
creased slightly even when the culture was held at 21°C for eight
days. A similar effect could be seen when the milk was treated after
inoculation but acid production by the cells was retarded under these
conditions. The intensity of this effect was proportional to the con-
centration of hydrogen peroxide used. This suggested it would be
possible to control the amount of diacetyl desired in a product by
regulating the amount of hydrogen peroxide used. This turned out to
be true to some extent, especially when the hydrogen peroxide-
catalase treatment was combined with the cooling process.

A level of 0.5 ppm diacetyl in beer was reduced to 0.1 ppm by
holding the beverage for three hours at room temperature with 20 mg
of crude diacetyl reductase and 8 mg of DPNH. These amounts of
enzyme and coenzyme could be reduced by adding alcohol dehydrog-
enase to the reaction system. However, the activity of these systems

were still low in beer with a pH of 4.3. Their effectiveness could be increased about 20-fold by adjusting the pH of beer to 5.0. From this it was concluded that the low pH of beer was the principal factor limiting the application of diacetyl reductase to control diacetyl off-flavor in beer.

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