AN ABSTRACT OF THE THESIS OF

Terry Norman Tolls (Name) for the M. S. (Degree) in Microbiology (Major)

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Title STUDIES ON CONTROL OF DIACETYL OFF-FLAVOR IN BEER

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Sporadic outbreaks of diacetyl off-flavor in beer are a serious economic problem to the brewing industry. Studies were carried out in an attempt to improve the understanding of the problem and to experiment with new ways of controlling this defect.

The Owades and Jakovac method of diacetyl determination as modified by Pack was further refined to increase its sensitivity to the low diacetyl levels encountered in beers. A survey of alcoholic beverages showed diacetyl levels of all samples tested to be below threshold levels for organoleptic detection. Comparing yeast strains on a per cell basis, a 2.5-fold difference was found to exist between yeast strains in their ability to produce diacetyl. Also, corn steep liquor addition to wort resulted in increased diacetyl production during the subsequent fermentation.

Diacetyl removal from beer was studied using both live whole cells and crude enzyme extracts. Cells of Streptococcus diacetilactis

18-16 destroyed diacetyl from solutions at a rate almost equal to that achieved by the addition of live,whole yeast cells. Yeast cells impregnated in a diatomaceous earth filter bed were found capable of destroying all of the diacetyl from solutions percolated through the bed. Undialyzed crude enzyme extracts from yeast cells removed diacetyl very slowly from beer at its normal pH. When attempted at a pH of 5. 0 or higher, rapid diacetyl removal was achieved. Dialyzed crude enzyme extracts from yeast cells were found to destroy diacetyl in a manner quite similar to that of diacetyl reductase from Aerobacter aerogenes, and both the bacterial extract and the yeast extract were stimulated significantly by the addition of NADH.

Diacetyl reductase was studied, and it was found that at least three strains of A. aerogenes were better sources of the enzyme than strain 8724, the strain generally studied. Gel electrophoresis results indicated that at least three different NADH oxidases were present in crude extracts of diacetyl reductase. Sephadex gel filtration was found to be an excellent method for separating NADH oxidase activity from diacetyl reductase activity. It was also noted that alcohol concentrations approximately equivalent to that found in beer were quite inhibitory to diacetyl reductase activity.

STUDIES ON CONTROL OF DIACETYL OFF-FLAVOR IN BEER

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF TABLES

STUDIES ON CONTROL OF DIACETYL OFF-FLAVOR IN BEER

INTRODUCTION

Diacetyl is considered to be a serious off-flavor in beer (9, 13, 68). The problem has become more serious, especially in the United States, with the modern trend to lighter and more mild beers. Because of this the levels of diacetyl which were previously not prominent, due to masking by other flavors, are now becoming more noticeable and less acceptable (8, 53). Changes in the composition of wort, acceleration of production, and attempts at continuous fermentation have all resulted in the more frequent occurrence of diacetyl off-flavor in beer.

Brewers have had some success in their attempts to control the diacetyl concentration in beer, but their methods are not always effective. Extended lagering of the beer is one means of diacetyl removal, but lengthy holding periods are not economically feasible due to the cost of additional storage facilities (37). Kräusening, the practice of pitching fresh yeast into fermented beer, is another means of diacetyl removal. This latter process, however, can lead to off-flavors in the finished beer due to yeast autolysis. Previous work in the Department of Microbiology at Oregon State University has demonstrated that various species of bacteria are capable of

destroying diacetyl in milk cultures (18, 19, 51). Seitz et al. (61) and Pack (49) demonstrated that a cell-free extract from one of these bacteria, Aerobacter aerogenes 8724, had diacetyl destroying properties attributed to the enzyme diacetyl reductase. They suggested the use of diacetyl reductase for the removal of diacetyl from beer. This suggestion prompted the present investigation of possible methods to control the diacetyl off-flavor in beer. Different approaches to the problem were attempted, such as the use of crude enzyme extracts added directly to the beer and the use of whole yeast cells in a filtration technique.

LITERATURE REVIEW

Occurrence of Diacetyl in Beer

Diacetyl in beer causes an off-flavor which has been described as "lactic-diacetyl, buttery, or sarcina-like" (68). In addition to beers, diacetyl is considered to be an off-flavor in wines (21, 52) and in citrus juices (6, 43). The cause of this problem and its extent has been reported on by many authors.

Causative Agents of Diacetyl Formation

Claussen (11) described the causative agent of beer's diacetyl problem to be a beer cocci belonging to the genus Pediococcus. Shimwell and Kirkpatric (63) studied diacetyl formation in beer and concluded that the causative agent was not in the genus Pediococcus but was a member of the genus Streptococcus. While it was known that bacterial infections such as that of pediococci produced diacetyl in beer, Burger, Glenister and Becker (8) reported that the yeast fermentation itself resulted in the production of some diacetyl as a fermentation by-product. They also claimed that Lactobacillus pastorianus, a common bacterial contaminant in beer during the lagering stage, produced diacetyl. Kato and Nishikawa (37) also said that "beer sarcina" (a term used synonymously with pediococci),

brewers' yeast and L. pastorianus all produced diacetyl in beer. Several lactobacilli capable of producing diacetyl in wine were reported on by Fornachon and Lloyd (21). Other species of bacteria capable of producing diacetyl in wine were described by Pilone, Kunkee and Webb (52). It was claimed by Drews et al. (17) that diacetyl could be formed by oxidation by yeasts as well as by bacterial infection. In addition to microbiologically formed diacetyl, beer exposed to air for prolonged periods of time at certain stages of processing yielded diacetyl formed non-microbiologically in the beverage (8).

Extent of the Diacetyl Problem

Methods for diacetyl determination differ widely in their specificity and sensitivity for diacetyl. Several references give excellent descriptions of methods of diacetyl determination (21, 37, 68, 69). A more recent method was described by Owades and Jakovac (47) and was later modified by Pack et al. (50). The differences between these various techniques have resulted in many discrepancies in the literature as to what level of diacetyl is normal in beer, what level is detectable organoleptically, and what level is objectionable as a serious off-flavor. West, Lautenbach and Becker (68) claimed that normal tasting beer was found to contain about 0.20 to 0.46 parts per million (ppm) of diacetyl. The level was said to

become organoleptically detectable at 0. 35 ppm and objectionable at approximately 0. 50 ppm. On the other hand, Burger et al. (8) reported that some of today's beers were found to be so mild that concentrations lower than 0. 35 ppm were organoleptically detected, but beers with normal taste and aroma were found to contain 0. 20 ppm of diacetyl. A survey conducted by Denshchikov, Rylkin and Zhvirblyanskaya (13) showed a diacetyl range of 0. 40 to 0. 96 ppm for six samples of Russian beer tested. Drews, Specht and Trenel (16) suggested that the diacetyl concentration in light beers should range between 0. 00 and 0. 07 ppm, and that the threshold level of detection was generally 0. 20 ppm. Of all the beers Drews and his co-workers tested, none were found to be above the threshold value. Compared to beers, wines were found to have much higher levels of diacetyl. Fornachon and Lloyd (21) presented a good discussion on diacetyl levels found in wines.

Brewing Techniques and Procedures

Fermentation Methods

Brewery fermentation methods vary considerably from plant to plant, and each brewmaster and his associates have their own ideas as to what method makes the best beer. In addition to consulting general references on beer production (22, 29, 45, 67), the

author was also aided by many other individuals in providing the following information in this regard (1, 4, 10, 64).

Barley malt, which when ground becomes a source of amylases and proteinases, is mixed with a cooked cereal adjunct (usually corn grits or rice). The mixing takes place in a "mash" tank (Figure 1) where the malt enzymes quickly go to work hydrolyzing the starches and proteins of the mixture. (At this point it is important to note that, depending on the region in which it is grown, different malts have dissimilar protein concentration levels. To adjust for these differences, some brewers incorporate a higher percentage of malt in their wort than other brewers.) As soon as the mixture has been hydrolyzed to the desired degree, the mashing temperature is raised to about 74 or 75°C to inactivate the malt enzymes. The liquid is then filtered through a filter pad of barley husks on a perforated stainless steel false bottom in a "lauter" tub. At this point the clarified liquid is referred to as "wort".

Hops are usually added at the rate of 0.75 pounds per barrel of wort, and the two are boiled together in a large copper or stainless steel "brew" kettle. Hops are added to give biological stability through their antiseptic value, to add flavor and aroma, and to improve the foaming properties and colloidal stability of the beer (2). The hopped wort goes through a hop strainer and enters the hot-wort tank. The wort is gradually cooled to yeast "pitching" temperature

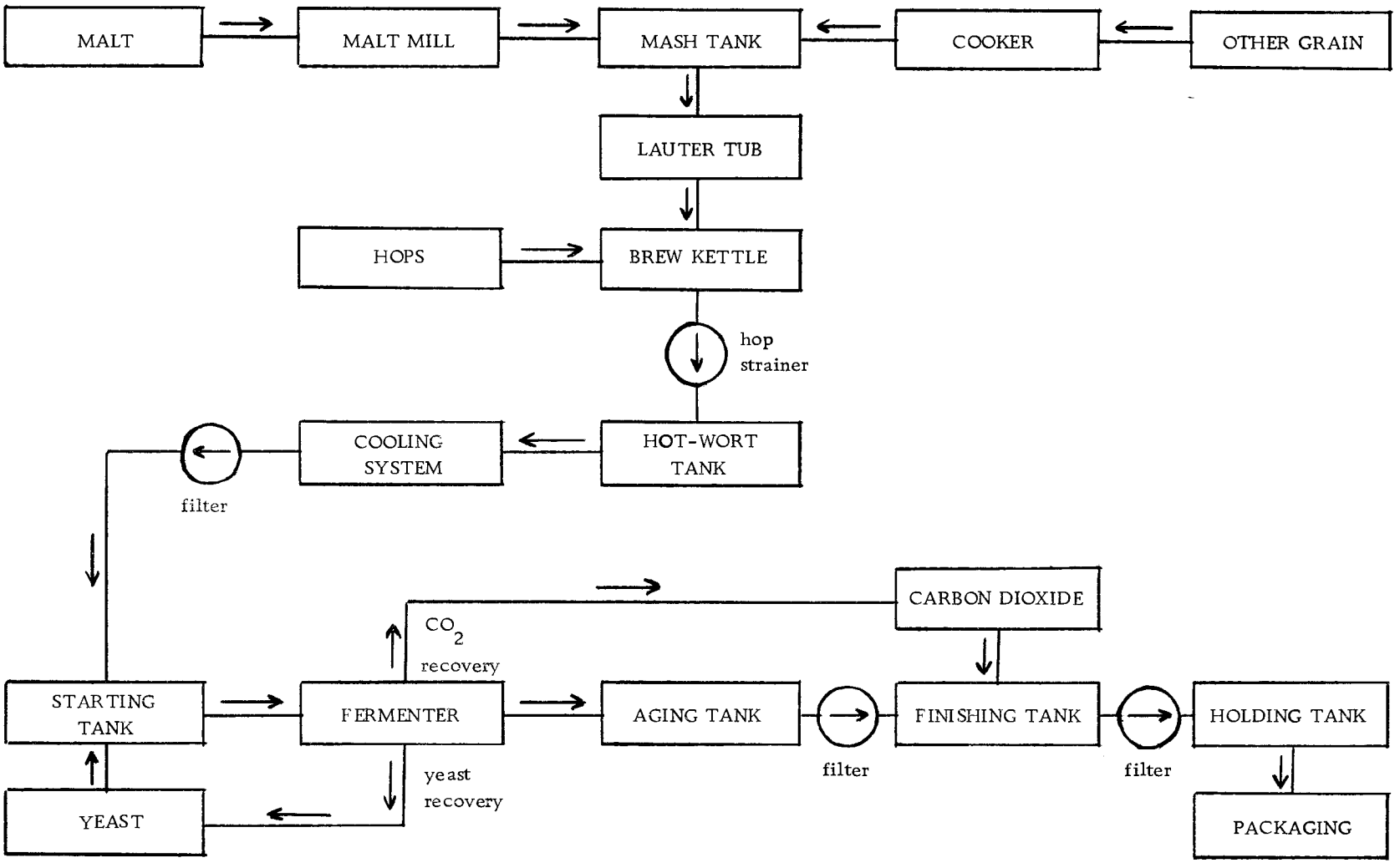

Figure 1. A typical brewery flow diagram.

which is about 10°C or lower. At this point the pH of the wort is generally between 5. 3 and 5. 5.

The wort is pumped into the primary fermentation tank (or the "starting" tank) and is inoculated with one to one and one-half pounds of yeast per barrel of wort; a barrel of wort contains 31 gallons and weighs 279 pounds. Here the inoculated wort is left from 12 to 24 hours to allow the fermentation to begin and to allow for some settling of the yeast and wort. Then the wort is pumped to the secondary fermentation tank (or the "fermenter") where fermentation is allowed to proceed. The wort temperature during fermentation differs in different breweries. It is usually in the range of 3. 3 to 15°C. Some brewers maintain the temperature at about 3. 3 to 4. 4°C during the entire fermentation, while other brewers start with relatively low temperatures and allow them to r i s e a few degrees as the fermentation proceeds. The length of the fermentation is dependent upon the temperature at which the fermentation proceeds, but the range is usually between 8 and 12 days. During fermentation the pH drops to about 4. 0, and then it rises to about 4. 1 or 4. 2. This rise in pH is probably due to yeast cell autolysis. The final alcohol content is around 3. 6 percent and is controlled by the amount of starch conversion allowed during mashing.

The fermented beer is then transferred to a primary storage tank (or the "aging" tank) where it is allowed to age. The aging

process lasts 10 to 30 days or longer while the temperature of the raw beer is maintained at 0°C so that settling will occur. As the beer is pumped from the aging tank to the secondary storage tank (or the "finishing" tank), the beer is passed through a diatomaceous earth filter bed. Here the beer remains for one, two, or more weeks. During finishing the carbon dioxide content of the beer is adjusted and final clarification steps are performed. The beer is then pumped through a final filter--the "polishing" filtration. This filter is a combination of diatomaceous earth and a filter pad. The beer is now sent to a holding tank and is subsequently packaged.

Beer filtration is of special interest to this study. The filtration of the beer leaving primary storage is done with a coarse grade of diatomaceous earth which allows a rather high flow rate. The polishing filtration of the beer, on the other hand, requires the use of a finer grade of diatomaceous earth. Here, acid-washed asbestos filter plates are coated with a one-sixteenth inch precoat of diatomaceous earth. These plates are placed in parallel and the beer, mixed with diatomaceous earth at the rate of one-tenth pound per barrel of beer, is distributed through baffleplates over the filter surface. The average flow rate of such an apparatus is about 225 barrels per hour through a 400 square foot surface. The peak flow rate is about 300 barrels per hour. The diatomaceous earth coat on each filter pad is approximately one-half inch thick at the completion

of the filtration process.

Choice of Yeast for Fermentation

In many cases, microorganisms that produce diacetyl are also capable of destroying it. A Betacoccus organism that was able to produce and destroy diacetyl at a rapid rate when grown at pH 4. 2 was described by Wiley (70). Seitz (58) was able to show that many of his dairy cultures produced high levels of diacetyl, but upon prolonged incubation the flavor compound was destroyed. Owades, Maresca and Rubin (48) claimed that diacetyl was both a product of and a substrate for the metabolism of yeast. They showed that diacetyl was produced during the aerobic stages of brewery fermentations and was partially destroyed during the anaerobic stage. The production and disappearance of diacetyl during a yeast fermentation was also described by Drews et al. (16) and by Murdock (43).

Many workers have shown that different yeast strains under otherwise identical conditions produced dissimilar levels of diacetyl (17, 37, 48, 53). These same yeast strains also differed considerably with regard to their diacetyl removing ability (17). However, Owades et al. (48) claimed that the differences in diacetyl production between the different yeast strains were largely eliminated by the end of the fermentation.

Juni (31, 32), although not associated with the brewing industry,

studied one portion of yeast metabolism and found that, in contrast to the bacterial system, the formation of acetoin by yeast does not involve alpha-acetolactic acid as an intermediate. He demonstrated by using pyruvic acid-2-C^{14} and unlabeled acetaldehyde as substrates, that the carbonyl carbon of acetoin came from the carbonyl group of pyruvic acid. Also, when non-labeled pyruvic acid and acetaldehyde-2-C^{14} were used as substrates, the acetoin produced was highly labeled in the carbinol carbon. However, he went on to say that several different mechanisms for the formation of acetoin in biological systems were possible.

As a result of a study on the nitrogen metabolism of yeasts, Owades et al. (48) were able to show that the presence of acetolactate or valine in the culture medium suppressed the synthesis of diacetyl by yeast cells. They found that the addition of 50 ppm of valine was not sufficient to effect diacetyl formation, but that the addition of 200 ppm or more had a marked depressive affect on diacetyl formation. It was also found that the addition of other nitrogen compounds had no depressive effect whatsoever. These studies led them to the conclusion that yeast cells produced diacetyl as a by-product of the synthesis of the amino acid valine. A mechanism proposed by Owades et al. (48) shows acetolactic acid as the precursor of both valine and diacetyl (Figure 2). Owades and his co-workers believed that both acetolactic acid and valine acted as feedback inhibitors to inhibit the

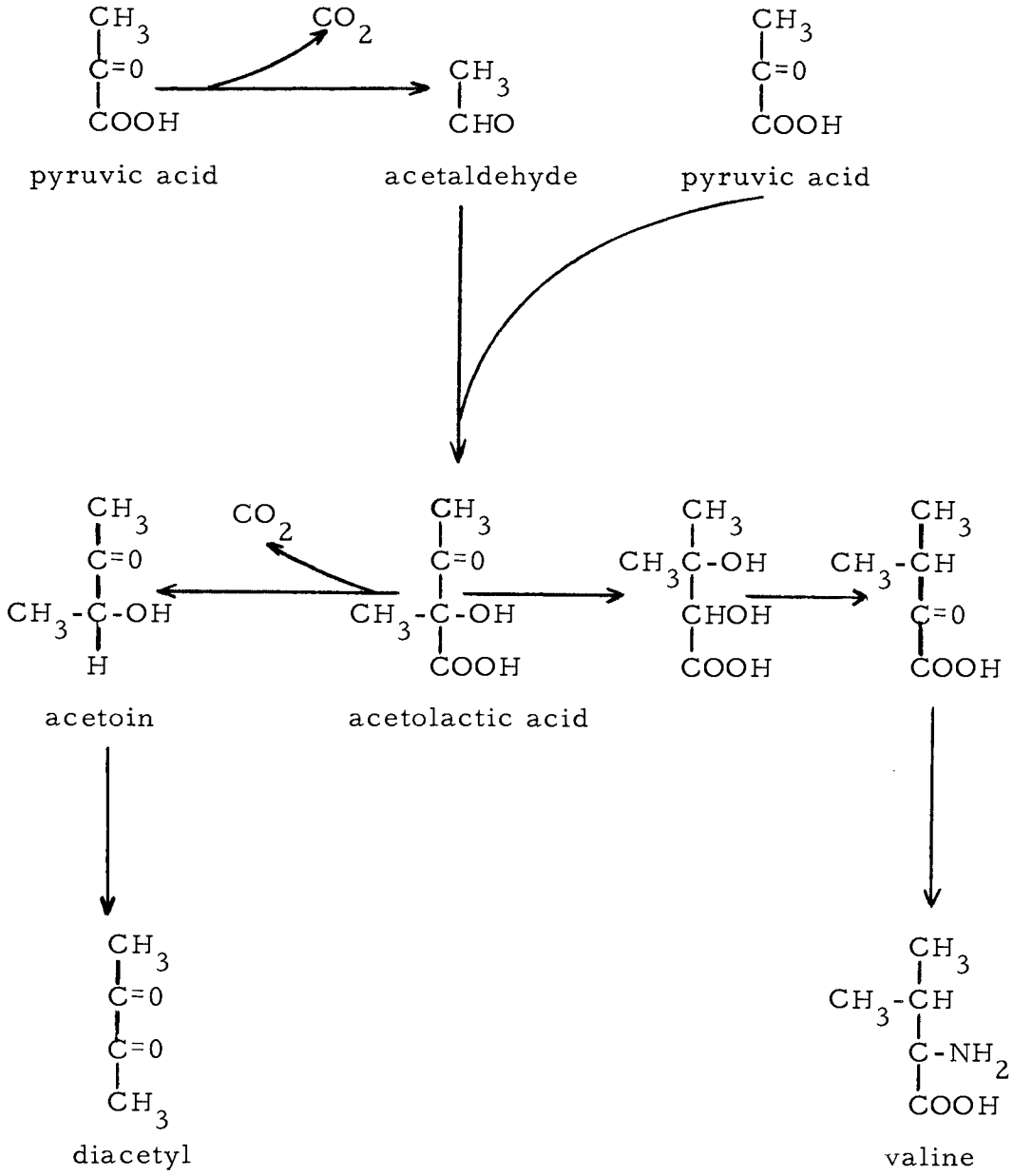

Figure 2. A mechanism of diacetyl formation as proposed by Owades et al. (48).

enzyme that formed acetolactic acid, the precursor of diacetyl.

Despite similar protein levels, Owades et al. (48) pointed out that the valine content of worts differed widely. Also, yeasts differed significantly in their ability to absorb valine from wort. Lewis and Phaff (40) demonstrated that yeast cells released measurable amounts of amino acids into their growth medium. It was shown that in the presence of excess sugar, the released amino acids were then reabsorbed by the cells in the course of a few hours (41). Stevens, in the discussion following the presentation by Brenner et al. (7, p. 245), indicated that at the diacetyl peak, the valine appeared to be completely utilized.

It has been suggested that yeast strain selection is possibly the most important single factor concerned with respect to the diacetyl problem (53). Owades et al. (48) suggested the use of a valine assay to test a yeast strain's ability to form diacetyl. However, the low final levels of diacetyl, due to the addition of valine, may only be the result of a delay in the onset of diacetyl formation during fermentation (53).

Physical Factors Affecting Diacetyl Concentration

Aerobic conditions were found to favor diacetyl production while anaerobic conditions tended to favor acetoin production, according to Burger et al. (8). Owades et al. (48) noted that any

treatment which invoked the Pasteur effect, such as the pitching of yeast into aerated wort, stirring, or transferring of wort during fermentation, was capable of directing the yeast toward diacetyl formation. The method of yeast preparation before pitching, according to Kringstad and Rasch (38), was found to have a considerable influence on the properties of the yeast during the subsequent fermentation. They noted that yeast prepared under constant stirring produced more diacetyl than yeast prepared statically.

Kringstad and Rasch (38) also claimed that pitching yeast prepared at 10°C produced less diacetyl than those propagated at 17°C. It was demonstrated by Portno (53) that fermentations occurring at elevated temperatures resulted in a higher diacetyl production than those at lower temperatures. As the fermentation temperature decreased, Anderson and Likens (2) claimed that the amount of soluble protein that remained in the wort increased.

When discussing a particular dairy culture, Cox (12) said that the rate of diacetyl synthesis and degradation was largely dependent on pH. A good discussion on the effect of pH on diacetyl production was presented by Pack (49).

Yeast cell concentration was found to have a considerable effect on diacetyl concentration (53). Wort pitched with a high concentration of yeast resulted in relatively high final concentrations of diacetyl. Drews et al. (16) noted that filtration and pasteurization

of beer had no effect on diacetyl and acetoin content.

Studies on Diacetyl Removal

Whole Yeast Cell Diacetyl Removal

According to Burger et al. (8), diacetyl off-flavor was removed from beer by refermenting it with about one-half its volume of fresh wort, and with yeast at the normal pitching rate. The addition of yeast at the normal pitching rate without the addition of fresh wort was also effective in removing diacetyl. This reducing power of the yeast was greatest during the period of intense yeast propagation (9). Even compressed bakers' yeast was capable of removing diacetyl; however, heat treated yeast cells were not capable of removing diacetyl.

Burger, Glenister and Lautenbach (9) also noted that the presence of fermentable matter was useful, in conjunction with yeast, in preventing the development of the diacetyl off-flavor.

Regardless of yeast species, Kato and Nishikawa (37) found that the addition of fresh yeast to beer to which diacetyl had been added was effective in the elimination of the diacetyl. They also showed that shaking the beer during the lagering stage increased the cell numbers in contact with the beer resulting in accelerated diacetyl removal. The ability of a yeast to remove diacetyl from beer

was shown by Burger et al. (9) to be directly related to the amount of yeast which remained in suspension in the beer during the period of treatment. Lagomarcino and Akin (39) added cells of Saccharomyces cerevisiae and studied factors affecting the removal of diacetyl from beer. They found that the diacetyl removal rate increased with increased temperature and yeast concentration and was faster when diacetyl concentration was greater.

It was suggested by Burger et al. (8, 9) that, even though the addition of yeast to fermented beer caused autolysis, yeast autolysis danger was minimized when the yeast was clean and when the beer was kept under carbon dioxide and at a temperature of about 0° C. Also, it was suggested that prolonged contact of the yeast with the beer be avoided.

Cell-free Crude Extract Diacetyl Removal

Green, Stumpf and Zarundnaya (27) isolated a "highly specific 'diacetyl mutase'" from pigeon-breast muscle which converted two molecules of diacetyl to two molecules of acetate and one of acetoin. The enzyme was found to be thiamine pyrophosphate (TPP) dependent and was quite stable except that it could not withstand lyophilization. It was suggested by Schweet et al. (56) that the diacetyl mutase reaction was not a true dismutation at all but was nothing more than pyruvic oxidase.

Diacetyl reductase, isolated from a strain of A. aerogenes, was first described by Strecker and Harary (66). In the presence of reduced nicotinamide adenine dinucleotide (NADH), this enzyme was able to reduce diacetyl to acetoin, and the reaction could be followed by measuring the rate of oxidation of the NADH at 340 mμ in a spectrophotometer. It was noted that this reaction was not reversible.

A cyclic pathway which involved diacetyl as an intermediate was proposed by Juni and Heym (34). This cycle, the 2, 3-butanediol cycle (Figure 3), was shown to provide a method for microorganisms to generate acetic acid. The enzymes concerned with the cycle were shown to be adaptive in nature. Cells grown in the presence of 2, 3-butanediol or acetoin were able to oxidize 2, 3-butanediol, acetoin and diacetyl while those grown in nutrient broth were not able to oxidize the above compounds. It was noted that the 2, 3-butanediol cycle showed no evidence of diacetyl oxidation via pyruvate oxidase or by the diacetyl mutase of Green et al.(27).

Juni and Heym (35, 36) studied the enzymes involved in the 2, 3-butanediol cycle. One of their findings was that several of the dehydrogenase reactions of the cycle were possibly catalyzed by the same enzyme. It was suggested that the reduction of diacetylmethylcarbinol be coupled with either lactic dehydrogenase or alcohol dehydrogenase as a means of maintaining a constant supply of the cofactor, NADH. They demonstrated that reduced nicotinamide

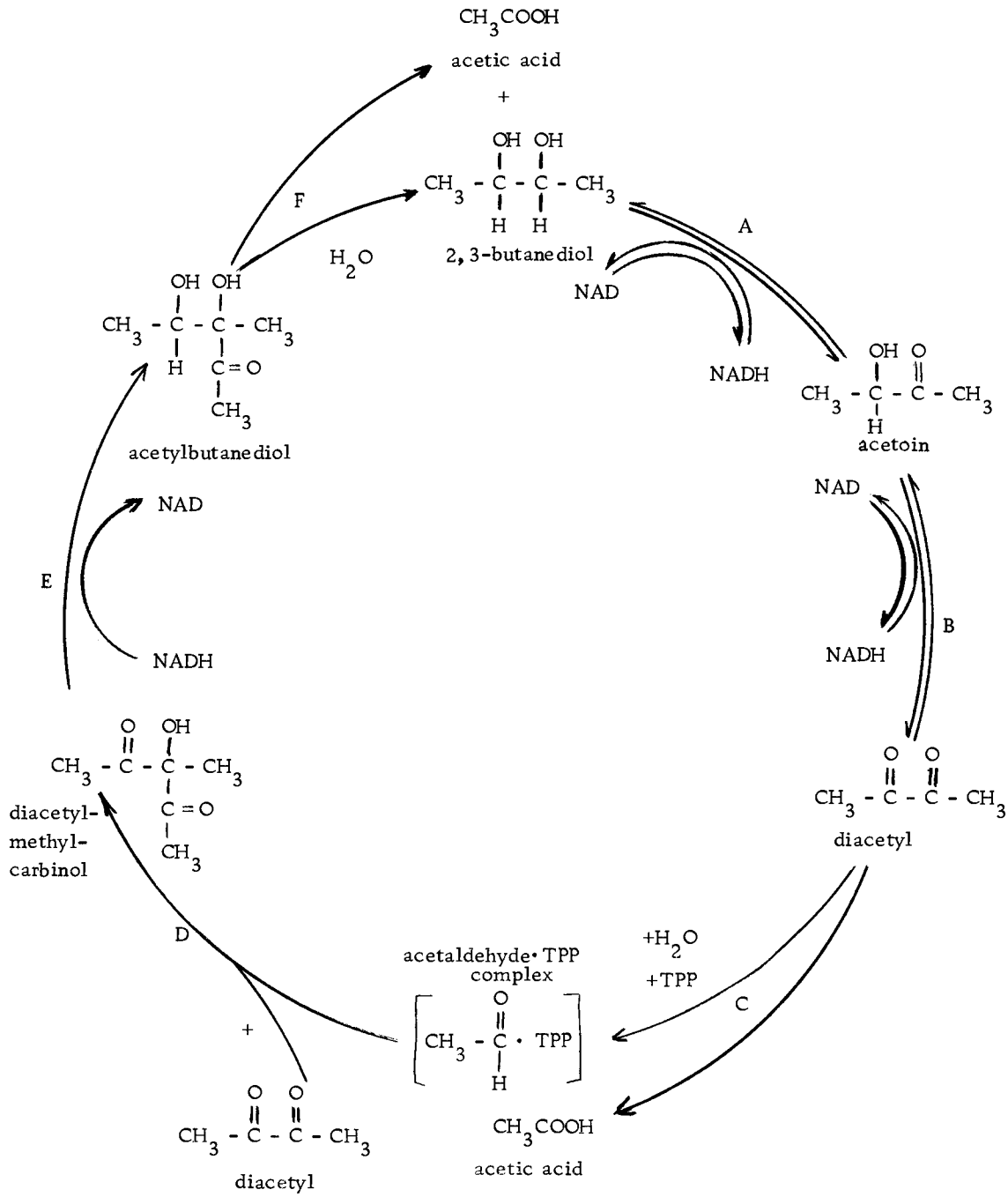

Figure 3. Reactions of the 2, 3-butanediol cycle as proposed by Juni and Heym (34, 35, 36).

adenine dinucleotide phosphate (NADPH) would not replace NADH as the reduced cofactor. Juni and Heym (36) reported that it had not been possible for them to demonstrate the presence of an enzyme concerned uniquely with the reduction of diacetyl in any of the micro-organisms which they studied. A. aerogenes formed different 2, 3-butanediol dehydrogenases when grown with glucose and acetoin as the carbon sources. Their investigations failed to furnish evidence for the existence of a diacetyl reductase enzyme distinct from 2, 3-butanediol dehydrogenase, nor were they able to show that acetoin was oxidized to diacetyl by their enzyme system. It was noted that an extract from bakers' yeast was found to contain a fairly strong 2, 3-butanediol dehydrogenase.

Most of Juni and Heym's work was done with a partially purified enzyme system (35). Still, it was noticed with some of their extracts that a small endogenous level of NADH oxidation was present due to "NADH oxidase". During enzyme assays this NADH oxidation had to be subtracted from the absorbance change observed when a substrate for the enzyme under study was added.

Cell-free crude extracts of yeast cells were prepared by Burger et al. (9), and different amounts of these extracts were used in an attempt to remove diacetyl from beer. All of their attempts failed even though the whole cells from which the extracts were prepared were capable of destroying diacetyl. Burger's group concluded

that the diacetyl destroying enzyme was tightly bound to the yeast cell material and therefore it was not found in the cell-free extract.

Seitz and his co-workers (59, 60, 61) claimed that the enzyme responsible for the loss of diacetyl in dairy products was probably diacetyl reductase. They demonstrated that the presence of diacetyl reductase was wide-spread among microorganisms used in dairy cultures and that it was especially important as a diacetyl destroying enzyme in psychrophilic microorganisms. Since the enzyme appeared to be non-reversible, Seitz (61) and Pack (49) suggested that it could possibly be used for the removal of diacetyl from beer.

The use of diacetyl reductase for the removal of diacetyl off-flavor in beer was experimented with by Bavisotto et al. (5). They came across two problems. First, the enzyme was effective down to a pH of 5.0, but below that the activity dropped off sharply. Second, the cost of the reduced cofactor was too high to make commercial use of the enzyme feasible. They experimented with coupling reactions as a means of maintaining a constant NADH concentration, but the pH factor was still a problem to the enzymes involved.

Recently, Drews and his co-workers (16, 17) showed that a hydrogen transfer enzyme found in yeasts, acetoin dehydrogenase, reversibly converted acetoin to diacetyl. The cofactor, nicotinamide adenine dinucleotide (NAD), was required.

Survey of Microorganisms for Diacetyl Reductase Activity

Many organisms have been found to be capable of destroying diacetyl. It was found that microorganisms capable of utilizing diacetyl, or related compounds, as a source of carbon frequently contained diacetyl reductase. Stanier and Fratkin (65) showed that a strain of A. aerogenes grown on a medium with acetoin as the sole source of carbon was able to oxidize 2, 3-butanediol, acetoin and diacetyl. A Corynebacterium was isolated from soil by Juni (30) that was able to use acetoin but not diacetyl. Also, it was found that when diacetyl was substituted in the medium, it would not support growth.

It is well known that diacetyl is toxic for many microorganisms at low concentrations (13, 23, 44). It is also known the acetoin, likewise, is toxic for some organisms (44). For example, Myrvik (44) showed that if a certain microorganism was sensitive to low concentrations of diacetyl, it was also sensitive to low concentrations of acetoin; however, it took about 50 to 80 times as much acetoin to inhibit growth as it did diacetyl.

Diacetyl reductase was found to be present in both Staphylococcus aureus and A. aerogenes by Strecker and Harary (66). An extensive study of microorganisms frequently encountered in the dairy field was made by Seitz et al. (61) to determine how many of them

had the enzyme diacetyl reductase. They found many positive and negative strains of each of the following: Streptococcus lactis, Streptococcus cremoris, Leuconostoc citrovorum, and Leuconostoc dextranicum. Other organisms they found to be positive included Streptococcus diacetilactis (six strains, all positive), A. aerogenes 8724, Escherichia coli OSU, Pseudomonas putrefaciens OSU, Pseudomonas fragi OSU, Pseudomonas fluoresens OSU, Pseudomonas viscosa OSU, and Alcaligenes metalcaligenes OSU.

Aubert and Millet (3) obtained a purified extract from Neisseria winogradsky which attacked diacetyl but not acetoin. Also, mycelial mats of Rhizopus nigricans were found to be able to convert diacetyl to acetoin, according to Fields and Scott (20).

Physical Non-enzymatic Methods for Diacetyl Removal

Since diacetyl is notably a highly reactive compound, not all reactions involving diacetyl as a reactant are necessarily catalyzed by enzymes. Schönberg (55) showed that when diacetyl was refluxed with phenylaminoacetic acid, a combination product was formed which in the presence of phenylhydrazine hydrochloride precipitated benzaldehyde phenylhydrazine.

It was shown by West et al. (68) that the diacetyl content of some beers lowered upon standing. They went on to say that since normal bottled beer was a reducing mixture, it was logical to conclude

that the diacetyl was gradually changed into other related compounds. Potassium metabisulfite and ascorbic acid, when added to beer, reduced the level of diacetyl present (8, 37). This practice, however, was not nearly as effective as the mere addition of yeast.

MATERIALS AND METHODS

Occurrence of Diacetyl in Beer

Diacetyl Determination

The colorimetric assay for diacetyl as originally described by Owades and Jakovac (47) and later modified by Pack et al. (50) was used in these studies. Employing this procedure, 12 tubes, each containing 20 ml of sample, were immersed in a 65°C water bath where they were flushed with nitrogen gas. The diacetyl was forced by the sweeping gas into a tube containing buffered hydroxylamine where the diacetyl was converted to dimethylglyoxime. The final pink color, measured on a Bausch and Lomb Spectronic 20 spectrophotometer, was the result of a complex formed between one molecule of ferrous sulfate and two molecules of dimethylglyoxime. The analysis was standardized by two methods. The first method utilized a solution of dimethylglyoxime added directly to the color reagents. The second method utilized a double distilled solution of diacetyl also added directly to the color reagents.

Survey of Alcoholic Beverages for Diacetyl Content

Beers, ales, malt liquors, champagnes and wines of several different commercial brands were obtained from a local grocer.

These beverages were tested for their diacetyl content both organoleptically and by the modified Owades and Jakovac method. The sensitivity of the modified Owades and Jakovac method was checked to determine how well low levels of diacetyl could be detected.

Brewing Techniques and Procedures

Cultures Used

Cultures used for this study (Table 1) were obtained from the stock culture collection of the Department of Microbiology, Oregon State University; from the American Type Culture Collection (ATCC), Washington, D. C.; and from Charles Pfizer & Company, Inc. All cultures were maintained either in yeast-complete-medium (YCM) (Table 2), in citrate broth (Table 3) or on wort agar (Table 4). Other media employed for particular experiments are indicated in the results section.

Diacetyl Production and Destruction Patterns

Two procedures for showing diacetyl production and destruction patterns were used. The first of these involved the preparation of two gallons of wort according to the composition shown in Table 5. The wort was then inoculated with one-fourth ounce of Fleischmann's yeast. Frequent agitation was used to hasten the start of the

Table 1. Cultures used

Microorganisms	Growth medium	Source
Saccharomyces cerevisiae var. *ellipsoideus*	Citrate broth	Dr. L. W. Parks
Saccharomyces cerevisiae 2091	YCM	Charles Pfizer & Co., Inc.
Saccharomyces cerevisiae 2000-3	YCM	Charles Pfizer & Co., Inc.
Saccharomyces cerevisiae I	YCM	Charles Pfizer & Co., Inc.
Saccharomyces cerevisiae 2094-N	Citrate broth	Charles Pfizer & Co., Inc.
Saccharomyces cerevisiae 1538	Citrate broth	Charles Pfizer & Co., Inc.
Saccharomyces cerevisiae 2094-P	YCM	Charles Pfizer & Co., Inc.
Saccharomyces cerevisiae PH3	Citrate broth	Charles Pfizer & Co., Inc.
Saccharomyces cerevisiae 2000	YCM	Charles Pfizer & Co., Inc.
Saccharomyces cerevisiae T	YCM	Charles Pfizer & Co., Inc.
Saccharomyces carlsbergensis	Commercial wort	Blitz-Weinhard Company
Bakers' yeast (caked)	---	"Red Star" brand
Bakers' yeast (dry granules)	---	"Fleischmann" brand
"C"-mold	Citrate broth	Isolated from 0.01 M citrate buffer
"R"-mold	Citrate broth	Dr. W. E. Sandine--isolated from milk
Pediococcus soyae 13621	Wort agar	ATCC

Table 1. Continued

Microorganisms	Growth medium	Source
Pediococcus cerevisiae 10791	Wort agar	ATCC
Acetobacter pasteurianus 6033	Wort agar	ATCC
Acetobacter melanogenus 9937	Wort agar	ATCC
Streptococcus diacetilactis 18-16	Citrate broth	Department of Microbiology, OSU, Stock Culture Collection
Aerobacter aerogenes 8724	Citrate broth	Department of Microbiology, OSU, Stock Culture Collection
Aerobacter aerogenes 8308	Citrate broth	Department of Microbiology, OSU, Stock Culture Collection
Aerobacter aerogenes 12658	Citrate broth	Department of Microbiology, OSU, Stock Culture Collection
Aerobacter aerogenes O. S. U.	Citrate broth	Department of Microbiology, OSU, Stock Culture Collection
Streptococcus faecalis O. S. U.	Citrate broth	Department of Microbiology, OSU, Stock Culture Collection
Streptococcus faecalis 10C1	Citrate broth	Dr. H. W. Seeley

Table 2. Composition of yeast complete medium (YCM)[a]

Ingredients	Grams per liter
Glucose	20
Tryptone	20
Yeast extract	10

[a]YCM agar was prepared by adding 15 grams of agar per liter of medium

Table 3. Composition of citrate broth[a]

Ingredients	Grams per liter
Tryptone	10
Glucose	10
Sodium citrate dihydrate	20[b]
Yeast extract	5
Dibasic potassium phosphate	1
Magnesium sulfate	1
pH 7.0	

[a]According to Sandine, Elliker and Hays (54).

[b]"Glucose broth" is identical in composition to "citrate broth" except that sodium citrate dihydrate was omitted.

Table 4. Composition of wort broth[a]

Ingredients	Grams per liter
Bacto-malt extract	15.00
Bacto-peptone	0.78
Maltose	12.75
Dextrin, Difco	2.75
Glycerol	2.35
Dipotassium phosphate	1.00
Ammonium chloride	1.00
pH 4.8	

[a]Wort agar was supplied by Difco Laboratories in an already prepared form. Wort broth was prepared using the formula of wort agar (14) but with the 15 grams of agar omitted.

Table 5. Wort composition used for laboratory brewing of beer[a]

Ingredients	Amount
Pabst Blue Ribbon malt extract	1 3-lb can
Sucrose	3 pounds
Fleischmann's dry yeast	1/2 ounce
Water	5 gallons

[a]According to Dr. A. W. Anderson.

fermentation. The fermentation was allowed to proceed for about seven days. The amount of diacetyl in the fermenting wort was determined at specific times by the modified Owades and Jakovac method using 20 ml samples at each time of sampling. The temperature was maintained between 14 and 16°C during the entire fermentation. At each sampling the pH of the wort was read using a Beckman Zeromatic pH meter.

For the second method, two 250-ml flasks of wort broth (Table 4) were inoculated with a five percent inoculum of actively growing S. cerevisiae 2091, a brewers' yeast strain. The temperature was maintained at 10°C and the flasks were shaken occasionally to hasten the start of the fermentation. The diacetyl concentration in the fermenting medium was determined at specific times as described above.

Yeast Strain Variations

Twenty ml quantities of wort broth (Table 4) were prepared in the large test tubes (25 by 250 mm--Corning No. 9820) used on the modified Owades and Jakovac apparatus. The same cultural and diacetyl determination methods described above were used to determine diacetyl production differences of eight brewers' yeast strains. The eight cultures used were S. cerevisiae strains 2000-3, I, T, 2094-N, 2091, 2000, PH3 and 1538. Standard plate counts were run

on the tubes tested at 63 hours of incubation.

Corn Steep Liquor Addition to Wort

Corn steep liquor was said to be a minor component of some brewery worts (1) and was said to be present in a concentration of approximately 60 ppm. The inositol (phytic acid) content of the corn steep liquor used in this experiment was about 1. 1 percent of the total composition of the liquor itself (Table 6). Therefore, every liter of commercial wort that contained the additive, contained 0. 06 ml of corn steep liquor which was equivalent to 0. 66 μg of inositol.

Table 6. Proximate analysis of corn steep liquor[a]

	Minimum	Maximum
Baume @ 60° F	28. 5°	30. 0°
Solids	50. 0%	52. 0%
Protein (percent nitrogen x 6. 25)[b]	22. 0%	24. 0%
Reducing sugars (calculated as dextrose)	5. 5%	7. 5%
Lactic acid[c]	7. 5%	11. 5%
Ash	8. 0%	9. 5%
pH	3. 7	3. 9

[a]According to the technical data sheet for "Staley's Regular Corn Steep Liquor" which was issued in February, 1954, by the A. E. Staley Mfg. Company, Decatur, Illinois.

[b]These are chiefly peptides, polypeptides, amino acids and ammonium salts. The amino nitrogen generally ranged from 1. 1% to 1. 5% of this amount.

[c]Lactic and phytic acids (inositol) are the two predominating non-nitrogen organic acids present. Phytic acid content generally ranges from 10 to 13% of this amount when isolated and calculated as phytin (a mixed calcium-magnesium salt of phytic acid). Therefore, about 1. 1% of the composition of corn steep liquor is phytic acid.

To one liter quantities of commercial wort, different levels of corn steep liquor and inositol were added. The levels of corn steep liquor added were equivalent to two times and six times the normal quantities used, while the level of inositol added to other flasks was equivalent to ten times the amount normally found in wort. Two sets of controls, inoculated and uninoculated, were also used. The inoculant was S. carlsbergensis, and the fermentation temperature was maintained at 7°C. Diacetyl concentration was determined at specific times as previously described.

Studies on Diacetyl Removal

Diacetyl Removal by Heat-Inactivated Cells Versus Live Cells

Whole cells were heat-inactivated by rapidly bringing a suspension of cells to 98°C in a test tube and then rapidly cooling the suspension to 25°C. Suspensions of live and heat-inactivated cells were incubated for a given length of time in the presence of diacetyl at 25°C in 0. 1 M potassium phosphate buffer at pH 7. 2. The percent of diacetyl removal was determined by using a modification of the procedure diagrammed on Table 7. Whole cell suspensions replaced the enzyme concentrations, and the NADH addition was omitted.

Table 7. A typical experimental design for the assay of crude enzyme extracts using the modified Owades and Jakovac apparatus[a]

Tube number	1	2	3	4	5	6	7	8	9	10	11	12
Buffer (ml 0.1 M KH_2PO_4)[b]	19	19	19	18	18	18	17.5	17.5	17.5	17	17	17
Enzyme (5 mg/ml)[c]	-	-	-	1	1	1	0.5	0.5	0.5	1	1	1
NADH (4 mg/ml)[d]	-	-	-	-	-	-	1	1	1	1	1	1
Diacetyl (20 μg/ml)	1	1	1	1	1	1	1	1	1	1	1	1

[a] Many variations of this experimental design were used.

[b] Beer was often substituted in the place of the buffer.

[c] Whole yeast cells, or other constituents, were frequently substituted in the place of the enzyme.

[d] NADH was not always included in this experiments.

Inactivation of Yeast Cells with Hydrogen Peroxide

An attempt was made to inactivate a suspension of Fleischmann's yeast by treating with different levels of hydrogen peroxide ranging from 0. 01 to 3. 5 percent. After 20 minutes of contact time with the suspension at room temperature, catalase was added to neutralize the residual hydrogen peroxide. The suspensions were then plated-out on YCM agar (Table 2) to determine the percent of cells in the suspension killed.

Diacetyl Removal Using Whole Yeast Cells in Dialysis Tubing

Fifty-ml quantities of a heavy suspension of Fleischmann's yeast were placed in cellophane dialysis tubing. The effect of this system on diacetyl concentrations in beer was studied. The level of diacetyl present in beer was raised to the desired level by the addition of diacetyl from a stock solution. The yeast cell suspension was washed several times with 0. 1 M phosphate buffer solution with a pH of 7. 2. The final washing of the cells took place in beer. Then the beer, with a pH of 4. 30, was incubated at 3. 6°C. Samples were taken at specified times and were tested for their diacetyl content using the modified Owades and Jakovac apparatus. In some cases the samples were also tested organoleptically by members of a taste panel.

Diatomaceous Earth--Yeast Cell Filtrations

Two different sizes of glass columns with coarse-porosity fritted glass filter discs were used in the following filtration experiments designed to study filtration of beer as a means of diacetyl removal. One column was 5 by 60 cm, while the other column was 12.5 by 14.7 cm. Each column was packed using a suspension of Johns-Manville Hyflosuper-cel (a commercial grade of diatomaceous earth used in beer filtrations). When sufficient diatomaceous earth suspension had settled out to the desired filter bed height, the remainder was poured off the top of the column. A refrigerated Gilson fraction collector was set to collect five ml of effluent liquid per tube. A solution of diacetyl was then passed through the column to determine the column's void volume. Diacetyl concentrations were determined using the modified Owades and Jakovac method.

A suspension of both diatomaceous earth and yeast cells was then prepared. The two yeasts used in these experiments were Fleischmann's yeast and S. carlsbergensis. With the Fleischmann yeast, 20 grams of dry yeast granules were mixed with 200 grams of diatomaceous earth in 2000 ml of distilled water (or beer). Since one gram of dry yeast is equivalent (on a per cell basis) to 2.5 grams of wet-packed yeast, 50 grams of the wet-packed brewers' yeast were mixed with 200 grams of diatomaceous earth to obtain

equal ratios of the constituents. The filter beds were prepared as described above. A diacetyl solution was then passed through the solution to determine the extent of diacetyl removal by the live yeasts impregnated in the column. Diacetyl concentrations were determined using the modified Owades and Jakovac method.

Bacterial and Mold Cell-free Crude Extract Preparation

Bacteria and molds were grown from a one percent inoculum in 2 to 40 liters of sterile medium for 24 hours at 30°C. Citrate broth (Table 3) was the medium most frequently used, but glucose broth (Table 3) and soil enrichment medium (Table 8) were also used.

Table 8. Composition of soil enrichment medium

Ingredients	Grams per liter
Ammonium phosphate	1.0
Magnesium sulfate	0.2
Potassium chloride	0.2
Diacetyl	10.0[a]
pH 7.2[b]	

[a]Other diacetyl concentrations used were 5.0 g, 1.0 g and 0.1 g per liter.

[b]A pH of 4.0 was also used which was adjusted with dilute HCl. For pH 7.2, the pH was adjusted with 0.1 M KH_2PO_4.

Following growth, the cells were harvested with the use of a continuous flow attachment for the Servall RC-2 refrigerated centrifuge at 12, 100 X g with a flow rate adjusted to approximately 300 ml per minute. The packed cells were recovered from the collection tubes by resuspension in 0. 1 M potassium phosphate buffer at pH 7. 2. The cells were washed three times in buffer and then resuspended in more buffer to a volume of 50 ml.

Crude enzyme extracts were prepared by disrupting the cells in a Raytheon 10KC sonic oscillator for 20 minutes. Cell debris was removed by centrifugation at 27, 750 X g for one and one-half hours in the refrigerated centrifuge. The supernatant was dialyzed against three, four-liter changes of distilled water, with each dialysis lasting eight hours. The crude enzyme was then lyophilized and stored in a deepfreeze until used. Protein determinations on the extract were done by the method of Lowry et al. (42).

Yeast Cell-free Crude Extract Preparation

Yeasts were grown from a one percent inoculum in two-liter amounts of sterile citrate broth (Table 3) and in sterile YCM (Table 2) for 24 hours at 30°C. In some cases, such as with Fleischmann's yeast, the yeast was used as supplied commerically and was not grown in citrate broth or in YCM.

Following growth, the cells were harvested with the use of the

large-capacity centrifuge head (GSA) of the Servall RC-2 refrigerated centrifuge at 4, 080 X g for ten minutes. The packed cells were recovered from the centrifuge tubes by resuspension in 0. 1 M potassium phosphate buffer at pH 7. 2. The cells were washed three times in buffer and then resuspended with sufficient buffer to make the suspension heavy, but still pipettable.

Ten ml of the heavy yeast cell suspension were added to the cylinder well of an Eaton cell press which had been prechilled to dry ice temperature. Leaving the cylinder in contact with the dry ice for 15 minutes more was sufficient to freeze the suspension. The piston was placed in the cylinder, and a pressure of 10, 000 pounds per square inch was applied by means of hydraulic press. The frozen suspension of cells was extruded through a small orifice in the bottom of the cylinder and was collected in a metal centrifuge tube. This material was thawed and then centrifuged at 27, 750 X g for one and one-half hours. At this point the supernatant was either used or it was dialyzed, lyophilized and stored in a deepfreeze until used. Protein determinations of the extract were done by the method of Lowry et al. (42).

Enzyme Assay of Cell-free Crude Extract Preparations

Enzyme activity assays were carried out by one of two methods. The first method involved the use of a recording spectrophotometer

which measured the rate of oxidation of the cofactor (NADH) necessary in the reduction of diacetyl. The second method involved the use of the modified Owades and Jakovac apparatus which was a colorimetric determination of the actual diacetyl concentration itself.

A continuous recording spectrophotometer (the Cary model 11 or the Gilford model 2000) was used to measure the activity of the crude enzyme extracts by following changes in the absorbance at 340 mμ caused by the oxidation of NADH. The reactions were initiated by the addition of diacetyl to solutions containing enzyme, NADH and buffer. A typical experimental design for the assay is shown in Table 9. After the blank was adjusted for zero absorbance, the initial absorbance following the addition of NADH was recorded. The diacetyl solution was then added to the cuvette, and the reaction was allowed to proceed at room temperature. The time in seconds (T) required for 50 percent reduction of the initial absorbance was used for the calculation of the enzyme units present. One unit of enzyme was defined as the amount of enzyme which caused a 50 percent reduction of the absorbance when $1/T = 10^{-6}$. For example, if an enzyme concentration of 0. 5 mg caused a 50 percent reduction in the initial absorbance in 50 seconds, then the reciprocal of the time, multiplied by 10^{6}, would equal 20, 000 units per 0. 5 mg or 40, 000 units per mg.

Table 9. A typical experimental design for the assay of crude enzyme extracts using a continuous recording spectrophotometer[a]

Constituents	Blank (ml)	Plus NADH (ml)	Complete (ml)
Buffer (0.1 M KH_2PO_4, pH 7.2)	2.9	2.7	2.6
Enzyme (5 mg/ml)	0.1	0.1	0.1
NADH (2 mg/ml)	-	0.2	0.2
Diacetyl (860 μg/ml)	-	-	0.1

[a]A table similar to this has been reported by Pack (49).

[b]The constituents are listed in the order in which they were added.

The second method involved the use of the Owades and Jakovac apparatus. It measured colorimetrically the amount of diacetyl destroyed at a given time and temperature for a given concentration of enzyme. Table 7 shows a typical experimental design for the assay of crude enzyme extracts by this method. Tubes one through three were used to determine the initial diacetyl concentration. Tubes four through six were used to detect enzymatic activity in the absence of the cofactor, NADH. Tubes seven through nine and ten through twelve were used to measure the enzyme activity for two different enzyme concentrations in the presence of the cofactor, NADH.

Effect of Lactate Dehydrogenase on Diacetyl Removal

Lactate dehydrogenase (Grade A) from rabbit muscle was obtained from Calbiochem in Los Angeles, California, in order to study its effect on diacetyl removal. An attempt was made to determine if this enzyme was capable of removing diacetyl in the same manner as that of diacetyl reductase from A. aerogenes. The cuvette was filled with 0. 5 mg of lactate dehydrogenase, 0. 4 mg of NADH, and 86 μg of diacetyl with sufficient potassium phosphate buffer to bring the volume to 3. 0 ml.

Effect of Alcohol on Diacetyl Reductase Activity

Diacetyl reductase from A. aerogenes was assayed according to the procedure described above. Various alcohol concentrations were obtained by direct addition of absolute alcohol to the reaction mixture in the cuvettes.

Separation of Diacetyl Reductase from NADH Oxidase

Several attempts were made to separate diacetyl reductase activity from the endogenous NADH oxidase activity found in cell-free crude extracts of A. aerogenes 8724. Thermal denaturation, ammonium sulfate fractionation, disc electrophoresis, and Sephadex gel filtration all were attempted.

Thermal Denaturation. An effort to destroy NADH oxidase activity by heat denaturation was made, based on the possibility that diacetyl reductase would be less heat labile than NADH oxidase. One ml quantities of a five mg per ml solution of the crude extract were pipetted into one ml capacity ampuls (Kimble--No. 12012-U) which were subsequently heat sealed. The ampuls were submerged in a water bath at 45° C. The temperature of the bath was increased at the rate of 3° C per minute. With each rise of 5° C, samples were pulled and were immediately cooled in an acetone-ice bath. The activity of the NADH oxidase and the diacetyl reductase was determined for each sample according to the procedure described using the continuous recording spectrophotometer.

Ammonium Sulfate Fractionation. Ammonium sulfate fractionation was used as a possible means of separating the two activities. With 4.1 M representing a saturated ammonium sulfate solution at 25° C (26), dilutions of this solution were used to obtain the percent saturation levels desired in this experiment. Each fraction was assayed for its diacetyl reductase and NADH oxidase content as described above.

Disc Electrophoresis. Disc electrophoresis, according to the method of Ornstein and Davis (46), was tried as a possible means of separating the two activities. The method employed involved the use of a three-layered polyacrylamide gel formed in small diameter

glass tubing. The large pore gel contained the sample, forming the first layer. The second layer was the same as the large pore gel in the first layer; however, no sample was added. This layer served to concentrate the sample. The third layer was the small pore gel. The samples were electrophoresed vertically for an arbitrary time after which they were removed from the electrophoresis apparatus and subsequently analyzed for their NADH oxidase and diacetyl reductase activities. Protein determinations were done with one percent amido schwarz stain in seven percent acetic acid. A seven percent acetic acid solution was used for destaining. A modification of the enzyme staining procedure suggested by Goldberg (24) and Goldberg and Cather (25) was used to detect the position of diacetyl reductase and NADH oxidase on the gel. The staining solution consisted of 8 mg of nitro-blue tetrazolium (NBT), 3 mg of NADH, 1.4 mg phenazine methosulfate (PMS), and 3 ml of a 860 ppm diacetyl solution dissolved in 7 ml of a 7.5 pH tris-HCl buffer. This was incubated for about one hour at 37°C to allow the color to develop. Colorless zones were indicative of either NADH oxidase activity or diacetyl reductase activity. Another modification of this same procedure was to mix diacetyl and NADH together and subject these to the gel for about one-half hour. The gels were then rinsed with distilled water. Phenazine methosulfate and NBT were added and the gel was incubated longer. This procedure was performed both in the presence and in the

absence of the substrate diacetyl. Photographs were taken of the results.

Sephadex Gel Filtration. A 2.5 by 45 cm Sephadex column was packed with type G-200 medium according to the technical information supplied by Pharmacia Fine Chemicals, Inc. A 0.1 M potassium phosphate buffer system was employed. Blue dextran 2000 was used to determine the void volume, and elution data were expressed as fraction numbers after the void volume was eluted. Four ml of a 40 mg per ml solution of the extract were added to the top of a 2.5 by 36 cm column. The concentration of the crude extract remaining in each of the 50 drop (1.35 ml) fractions eluted from the column was followed by absorbance readings at 280 mμ with a Gilford model 2000 spectrophotometer. These fractions were then assayed for both their NADH oxidase activity and their diacetyl reductase activity.

Miscellaneous Experiments

During the course of this study, several experiments were carried out which did not provide meaningful data or information which would lead to subsequent work. For completeness, the following paragraphs list several of these experiments.

A soil enrichment medium (Table 8) was used in an attempt to isolate an organism which could utilize diacetyl as a sole source of carbon. Soil was added directly to the medium which was maintained

in screw capped flasks to prevent heavy loss of diacetyl.

Experiments were carried out to determine whether diacetyl reductase was related to Green's diacetyl mutase (27) (i. e. pyruvic oxidase (56)). Substituting diacetyl as the substrate, an experiment was designed similar to Dolin's (15) experiment on diacetyl oxidation by S. faecalis.

Three experiments performed by Pack (49) were repeated. The first experiment was to demonstrate the effects of ethylenediamine-tetracetic acid (EDTA) dialysis on diacetyl reductase. The second experiment was to show that alcohol dehydrogenase could be used in coupling experiments with diacetyl reductase. The third experiment was an attempt to reverse the activity of diacetyl reductase.

An attempt was made to destroy diacetyl by a non-enzymatic means using a modification of the assay shown on Table 7. Tryptone, casamino acids, histamine, glutamine and lysine, all were incubated for differing lengths of time in the presence of buffer and a mild diacetyl solution. The amount of diacetyl removal was determined on the modified Owades and Jakovac apparatus.

RESULTS

Occurrence of Diacetyl in Beer

Table 10 demonstrates the ability of the modified Owades and Jakovac method to differentiate between low levels of diacetyl. This table illustrates the approximate degree of deviation that can be expected when triplicate determinations of the same sample are made. It was found that 95 to 98 percent of the diacetyl added to beer was recovered by this method.

Table 10. Triplicate absorbance readings at 530 mμ representing low levels of diacetyl collected from a water system by the modified Owades and Jakovac method

ml solution[a]	Absorbance at 530 mμ 1	2	3	Average ppm diacetyl
0.00	.005	.005	.001	0.02
0.10	.016	.018	.018	0.08
0.20	.039	.037	.038	0.17
0.30	.058	.053	.057	0.25

[a]One ml of solution, when brought to a total volume of 20 ml with distilled water, was approximately equal to 0.8 ppm of diacetyl.

Of the alcoholic beverages tested for their diacetyl content (Table 11), it is important to note that none of the malt liquor, ale, or beer samples were above the low threshold level of 0.20 ppm

Table 11. Survey of alcoholic beverages for diacetyl content

Beer		Wine		Malt liquor and ale		Champagne	
Sample	Diacetyl (ppm)	Sample	Diacetyl (ppm)	Sample	Diacetyl (ppm)	Sample	Diacetyl (ppm)
A	0. 07	A	0. 33	A	0. 14	A	0. 26
B	0. 15	B	0. 20	B	0. 12	B	0. 24
C	0. 08	C	0. 37	C	0. 07	C	0. 28
D	0. 07	D	0. 18	D	0. 07		
E	0. 05	E	0. 25	E	0. 02		
F	0. 05	F	0. 30	F	0. 04		
G	0. 06	G	0. 45	G	0. 14		
H	0. 16	H	0. 17				
I	0. 09	I	0. 23				
J	0. 06	J	0. 31				
K	0. 04	K	0. 27				
L	0. 15	L	0. 37				
M	0. 10	M	0. 28				
N	0. 08	N	0. 33				
O	0. 11	O	0. 36				
P	0. 07	P	0. 22				
Q	0. 05	Q	0. 27				
R	0. 13	R	0. 35				
S	0. 08	S	0. 11				
T	0. 12	T	0. 14				
U	0. 05						
V	0. 04						
W	0. 06						
X	0. 09						
Y	0. 08						
Z	0. 10						
AA	0. 04						
BB	0. 07						
Range	0. 04 to 0. 16		0. 11 to 0. 45		0. 02 to 0. 14		0. 24 to 0. 28
Average	0. 08		0. 27		0. 09		0. 26

described by Drews et al. (16). The levels of diacetyl found in the wines and the champagnes were higher than those found in the malt beverages. Organoleptic tests of the beverages were not too conclusive. However, it was noticed that the taste panel members were in agreement on the relative diacetyl content of many of the samples.

Brewing Techniques and Procedures

Figure 4 shows a diacetyl production and destruction curve typical of yeast fermentations at 14 to 16°C. The pH of the wort indicates the extent of the fermentation. The lower the temperature, the longer each fermentation required to reach its peak diacetyl concentration. Figure 4 shows the peak was achieved after 48 hours of incubation at 14 to 16°C. Figure 5 shows that after 96 hours of incubation at 10°C, the peak was reached; while the uninoculated control in Figure 6 demonstrates that even after 189 hours, the diacetyl peak at 7°C had not been reached. Figure 4 further demonstrates that once the diacetyl peak was achieved, the diacetyl concentration continued to decrease as long as the yeast cells were left in contact with the beer. One batch of beer, prepared with the medium of Anderson (Table 5), was bottled after the sixth day of the fermentation. After two more weeks of storage at 20°C, the yeasts settled to the bottom of the bottle and the diacetyl concentration was found to have disappeared completely.

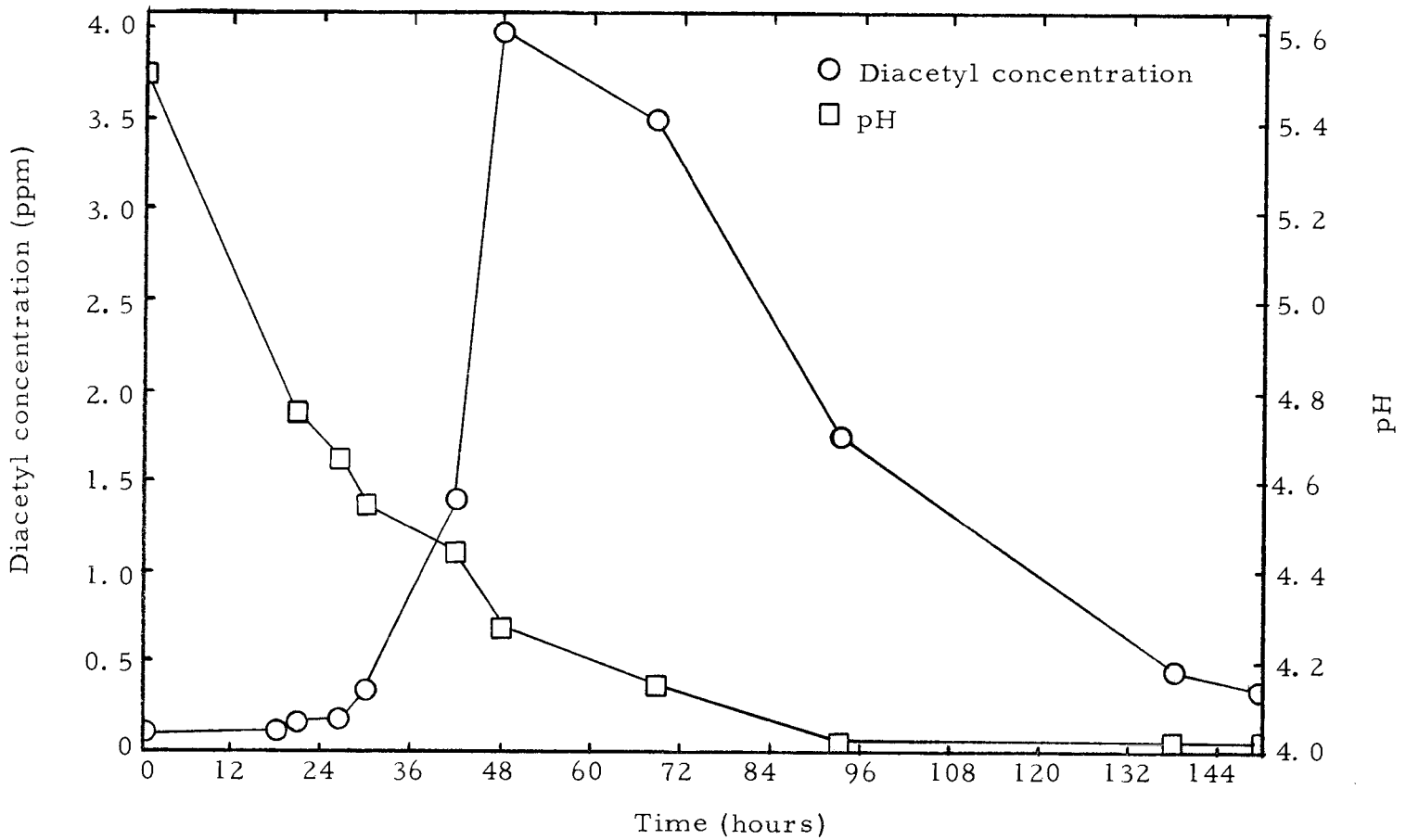

Figure 4. Diacetyl produced and pH attained at 14 to 16°C by Fleischmann's yeast after different times of incubation in the medium of Anderson (Table 5).

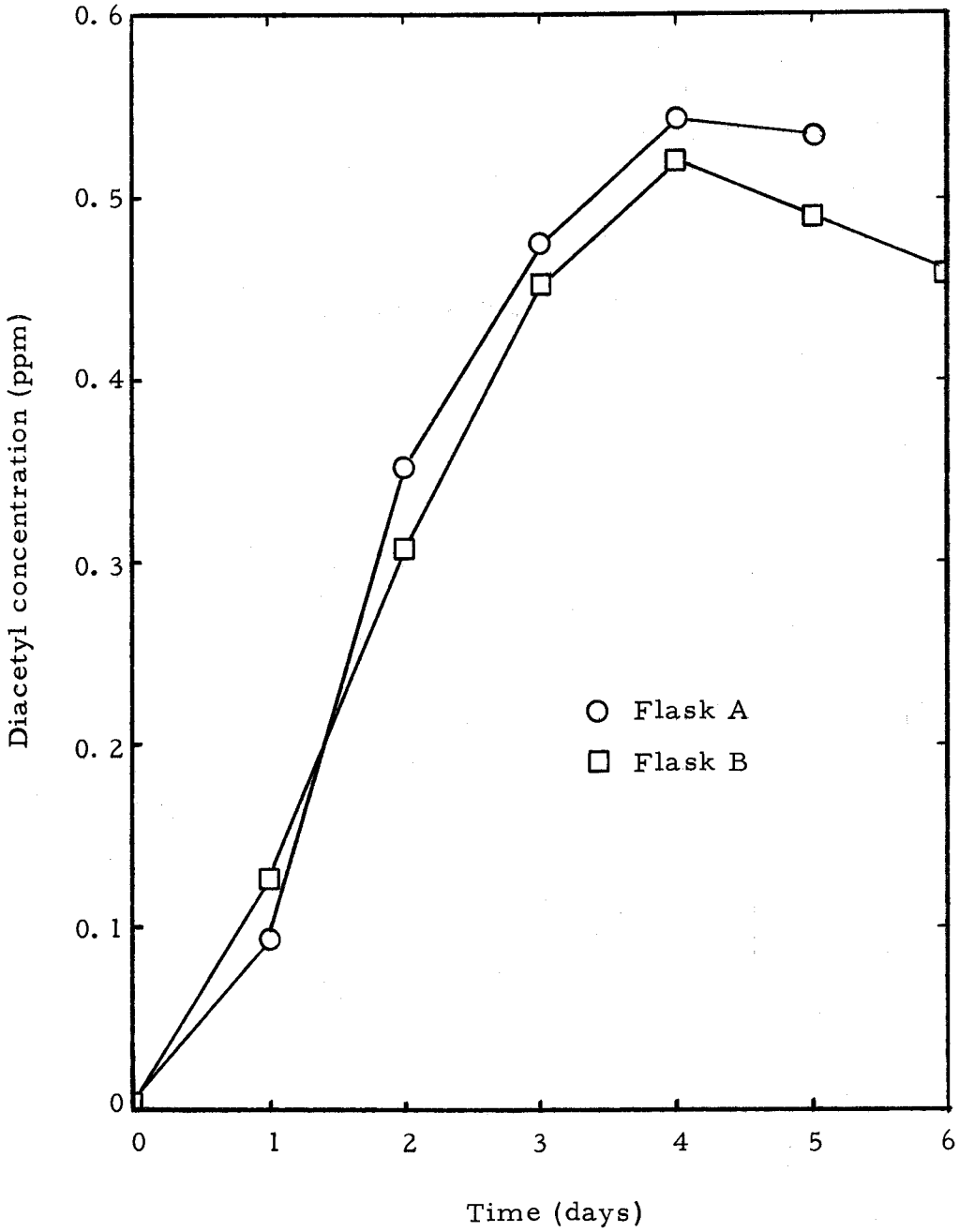

Figure 5. Duplicate determinations in flasks A and B of diacetyl produced by S. cerevisiae 2091 incubated at 10°C in wort broth for the times indicated.

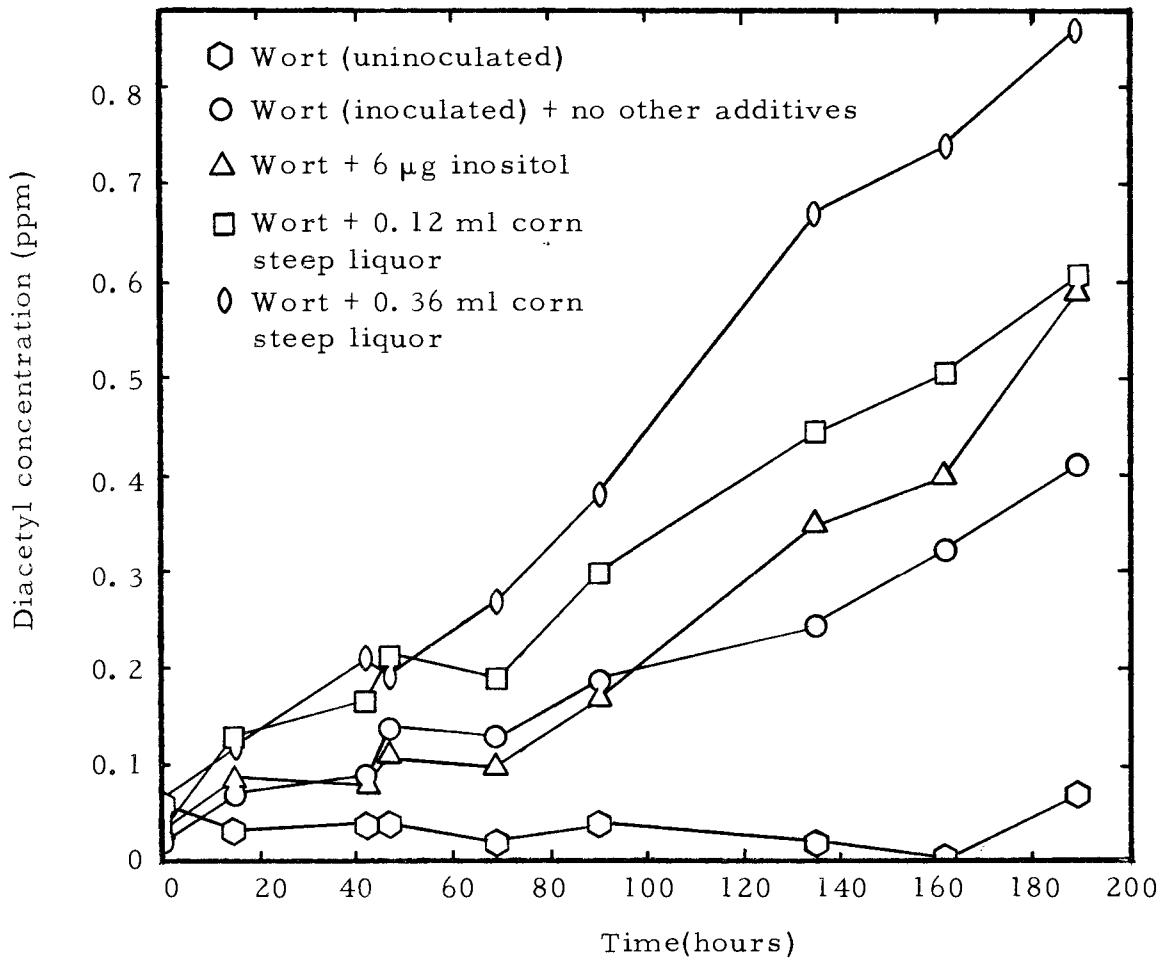

Figure 6. Effect of corn steep liquor and inositol additions to commercial wort on the amount of diacetyl produced by S. carlsbergensis when incubated at 7°C for the times indicated.

Duplicate determinations, for separate fermentations in flasks A and B, of diacetyl produced by S. cerevisiae 2091 are shown in Figure 5. The flasks were incubated at 10°C in wort broth, and samples were analyzed daily. The results of the graph are discussed above. Eight yeast strains were grown in a duplicate set of tubes under identical conditions. One set was checked for diacetyl content at the end of 63 hours of incubation while the other set was checked three hours later. A standard plate count was run on the 63 hour tubes. Table 12 relates the amount of diacetyl produced by these yeast strains with one another. Strain 2000-3 showed poor growth in this medium. The plate count data for this strain was undetermined.

The addition of corn steep liquor to commercial wort resulted in an increase in the amount of diacetyl produced during the fermentation (Figure 6). S. carlsbergensis, in the presence of commercial wort without added corn steep liquor, was found to produce about 0.4 ppm of diacetyl after approximately eight days. When two times the normal quantity of corn steep liquor was added, after eight days, 50 percent more diacetyl was produced than in the original fermentation. When six times the normal amount was added, the yeast produced better than 80 percent more diacetyl than in the original fermentation. When ten times the normal inositol content of corn steep liquor was added to the wort, about 35 to 40 percent more diacetyl

Table 12. Cell population and diacetyl produced by various strains of S. cerevisiae determined after incubation at 10°C in wort broth for 63 and 66 hours

Yeast strain	Plate count[a]	Diacetyl concentration (ppm) at 63 hrs	at 66 hrs	at 63 hrs (per cell x 10^9)	Relative diacetyl production[b]
Control	---	0.08	0.05	--	--
2000-3[c]	undetermined	0.10	0.08	--	--
2000	1.70×10^7	0.54	0.57	32	1.00
1538	1.18×10^7	0.59	0.60	50	1.56
PH3	1.73×10^7	0.62	0.63	36	1.12
2091	8.00×10^6	0.63	0.65	79	2.47
I	1.25×10^7	0.70	0.82	56	1.75
T	1.17×10^7	0.79	0.85	67	2.09
2094-N	1.83×10^7	1.00	1.10	55	1.72

[a]Plate counts were run on tubes incubated for 63 hours only.

[b]Relative diacetyl production is figured on the basis that the amount of diacetyl produced by strain 2000 is unity.

[c]Strain 2000-3 showed poor growth in this medium.

resulted.

Studies on Diacetyl Removal

The effect of using live, whole cells and heat-inactivated cells to remove diacetyl from an aqueous solution is shown in Table 13. The use of Fleischmann's yeast resulted in the greatest diacetyl removal, with 90 percent of the initial diacetyl concentration removed. The mixture of brewers' yeast strains resulted in the removal of 75 percent of the initial diacetyl concentration. Even S. diacetilactis 18-16 was capable of removing 70 percent of the initial diacetyl concentration. Heat-inactivated cells of each of the above suspensions were not capable of destroying even the slightest amount of diacetyl.

Attempts were made to inactivate suspensions of Fleischmann's yeast with various concentrations of hydrogen peroxide. A suspension of the hydrogen peroxide treated cells would then be tested for their ability to remove diacetyl. The hydrogen peroxide, at the concentrations employed, was ineffective against the yeast cell suspensions. Upon the addition of the hydrogen peroxide, severe foaming occurred, but plate counts revealed that no inactivation of the cells had occurred.

The diacetyl destroying property of live yeast cells was utilized in the two following experiments. The first of these experiments utilized a heavy suspension of Fleischmann's yeast contained in

Table 13. The ability of live whole cells and heat-inactivated cells to remove diacetyl from an aqueous solution buffered at pH 7. 2 at a temperature of 25°C

Microorganism	Cell concentration[a] (g/tube)	Reaction time (hours)	Diacetyl concentration (μg/tube)	Percent diacetyl removed	
				Heat-inactivated cells	Live cells
Brewers' yeast[b]	0. 24	2 3/4	30	0	75
Bakers' yeast[c]	0. 24	2 3/4	30	0	90
S. diacetilactis 18-16	0. 27	3	30	0	70

[a]Cell weight was based on wet-packed cells.

[b]*S. cerevisiae* 2000, I, and T were mixed in equal amounts.

[c]Fleischmann's yeast.

dialysis tubing. The other experiment involved the use of live yeast cells (Fleischmann's yeast and S. carlsbergensis) impregnated in a diatomaceous earth filter bed. In the case of the first experiment, diacetyl was removed from beer by yeast cells contained in a dialysis tubing (Figure 7). The beer was at its normal pH (pH 4. 30) and the temperature of the experiment was maintained at 3. 6°C. Flavor panel results of one such experiment indicated that extensive yeast autolysis had occurred during the three day reaction time. Nevertheless, the diacetyl level of the beer was decreased using this technique.

The second method, which employed the diatomaceous earth filter beds, successfully destroyed all the diacetyl in the solutions tested, and the yeast autolysis odor was no longer a problem. Figure 8 compares the ability of two different filter beds to destroy diacetyl. Using a 5 by 18 cm diatomaceous earth filter bed, it was found that the first traces of a diacetyl solution percolating through the bed were recognized at fraction number 43. The column with the yeast cells impregnated in the diatomaceous earth allowed no diacetyl to penetrate. The flow rate of the column was approximately 12 drops per minute. Faster flow rates were obtained with a larger column. The filter bed of the new column used was about 2 cm deep. As a result of the increased surface area and the shallower bed, the flow rate was faster than the Gilson fraction collector was able to count.

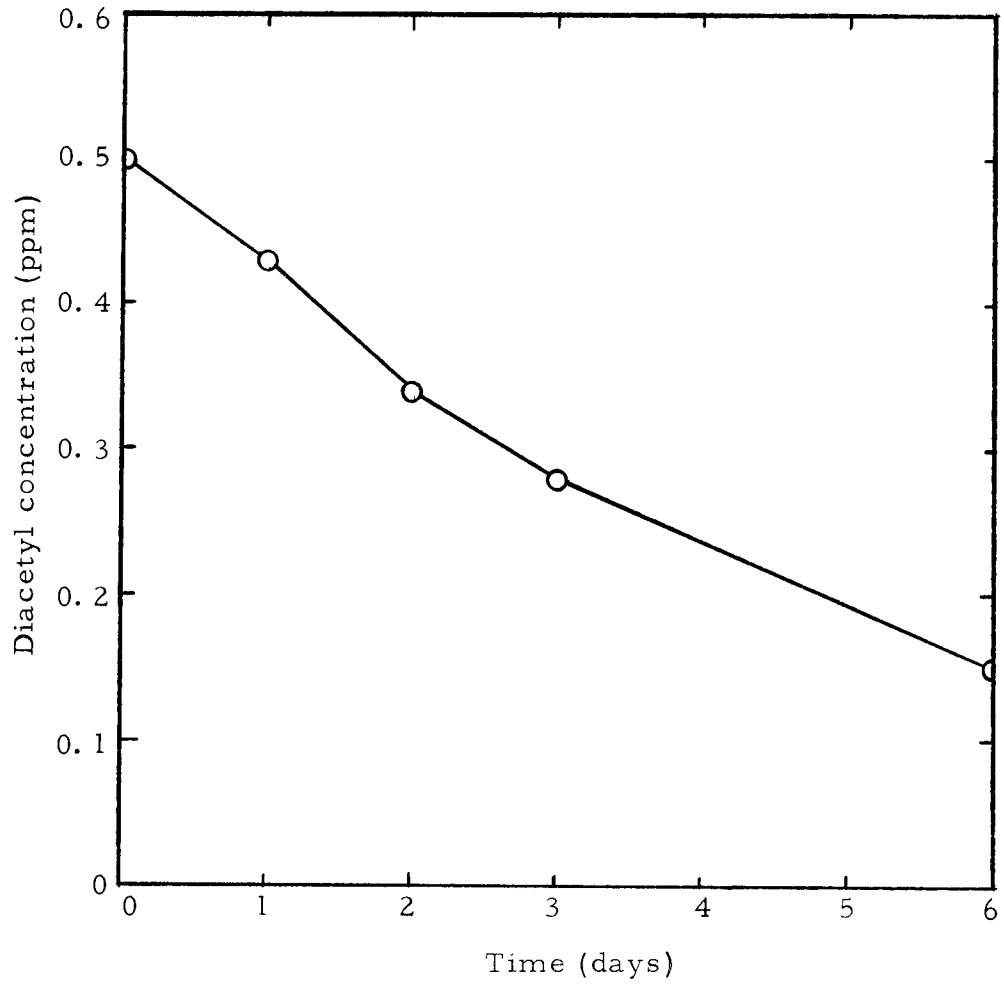

Figure 7. The ability of 50 ml of a heavy suspension of live, whole yeast cells contained in dialysis tubing to remove diacetyl from beer at its normal pH and at a temperature of 3.6°C.

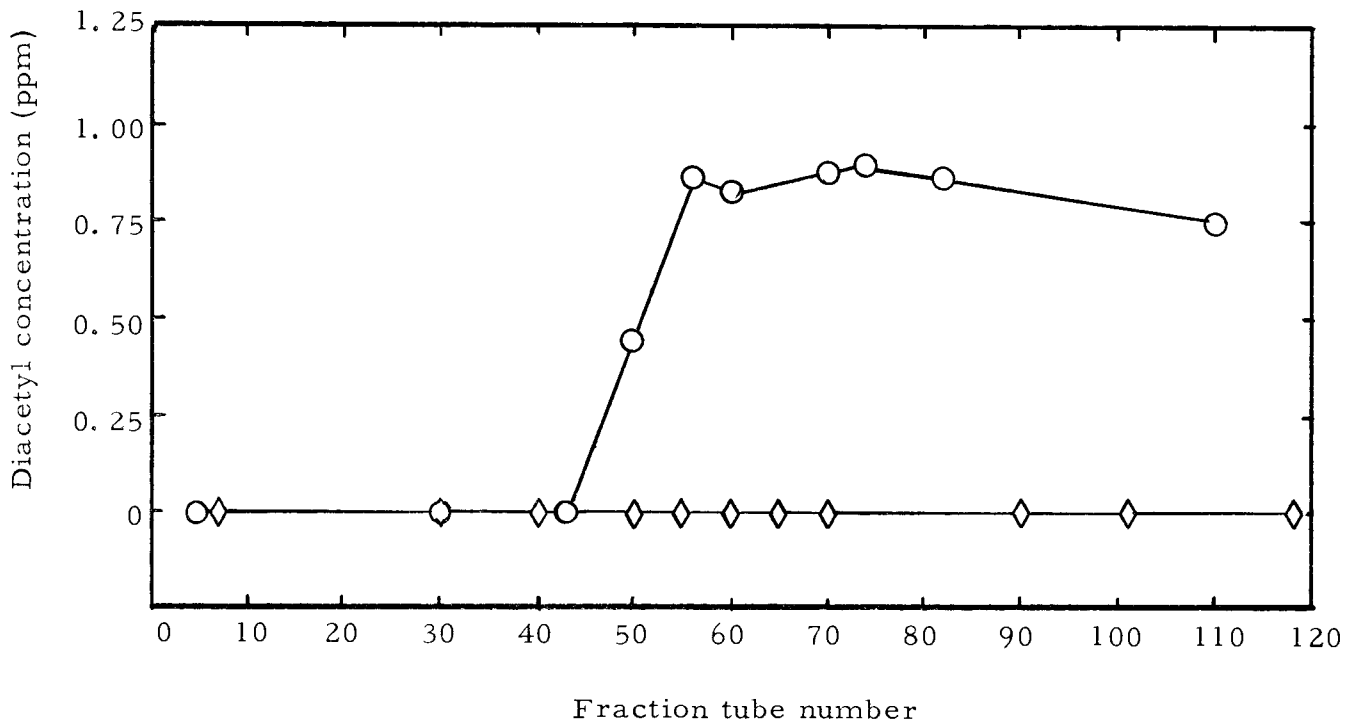

Figure 8. Comparison of the ability of diatomaceous earth (- O -) and diatomaceous earth impregnated with yeast cells (- ◊ -) to prevent the penetration of diacetyl through each of the 18 cm deep filter beds.

The flow rate had to be adjusted down to about five drops per second. Here again, all of the diacetyl was destroyed.

Crude enzyme extracts from bacteria and molds were easily prepared by sonication of the cells, but yeast cells were found to be too resistant to sonication to justify its use. Other means for crude enzyme extract preparation were attempted, such as the use of a mortar and pestle to grind cells in the presence of alumina, the incubation of the yeast cells in one percent glucose before sonication, and the use of the Eaton press to disrupt the cells. The latter method was found to be extremely effective. Table 14 contains data that shows the ability of the Eaton press to disrupt heavy suspensions of Fleischmann's yeast. It should be noted that when the supernatant liquid was decanted from the packed cells, a certain number of live whole cells always remained in the liquid. To make certain that the diacetyl destroying activity of the extract was not caused by these remaining cells, a suspension of approximately 1000 live,whole cells per ml was tested using the modified Owades and Jakovac method, for its ability to destroy diacetyl. After 30 minutes at 30°C, no diacetyl was destroyed by the suspension. Also, a suspension of cell debris containing about 6000 live,whole cells per ml was found to have no diacetyl destroying ability.

Table 14. Data showing the ability of the Eaton press to disrupt a heavy suspension of Fleischmann's yeast

	Trial 1[a]	Trial 2[b]
Original cell suspension[c] (cells/ml)	1.99×10^9	1.99×10^9
Disrupted cell suspension (cells/ml)	1.26×10^7	9.7×10^6
Percent kill	99.04	99.50
Cells in extract[d]	6.3×10^3	6.1×10^3

[a]Frozen 15 minutes after addition of suspension.

[b]Frozen 45 minutes after addition of suspension.

[c]Cell counts were made in duplicate on YCM at 30° C for 36 hours.

[d]The disrupted cell suspension was centrifuged and the supernatant liquid was decanted off the packed cells. This shows the number of live cells remaining in the extract after one centrifugation.

Initial attempts to demonstrate that crude enzyme extracts of yeasts were capable of removing diacetyl from beer were not too encouraging. Table 15 shows data concerning the ability of undialyzed extract of Fleischmann's yeast to remove diacetyl from beer. At a pH of 4.30 and at a temperature of 5° C, in 64 hours, 42 mg of extract were able to destroy approximately nine percent of the diacetyl from a 1.10 μg per ml initial concentration. The low pH of the beer caused much of the crude enzyme extract to precipitate. At this pH with a reaction time of two hours, even at a temperature of 30° C,

Table 15. Ability of undialyzed crude enzyme extract of Fleischmann's yeast to remove diacetyl from beer incubated at the times and temperatures indicated[a]

Beer sample[b]	Crude enzyme extract (mg/ml)	Reaction time (hours)	pH[c]	Temperature	Initial diacetyl concentration (mg/ml)	Diacetyl removed (%)
A	21	2	4.35	30°C	1.31	0
B	42	2	4.35	30°C	1.31	0
C	42	64	4.30	5°C	1.10	9

[a]Diacetyl was added to increase the diacetyl concentration to the levels desired for this experiment.

[b]Each sample indicated represents the average of several tubes.

[c]In each case, after a short time, the low pH caused the enzyme extract to precipitate out.

no measureable diacetyl destruction was apparent. When, as shown in Figure 9, the pH of the beer was raised with sodium hydroxide to 5.25, essentially the same concentration of the extract removed more than 80 percent of the diacetyl at 30°C in one hour. It was also shown that as the concentration of the undialyzed crude enzyme extract from Fleischmann's yeast was increased, the amount of diacetyl removed in 45 minutes at pH 7.2 and at 30°C increased. Figure 10 shows the linear relationship between diacetyl removal and enzyme concentration. The addition of a heavy suspension of heat-inactivated Fleischmann's yeast cells to the crude enzyme extract had no effect on the ability of the extract to destroy diacetyl.

The dialyzed crude enzyme extract of Fleischmann's yeast was found to have diacetyl destroying properties similar to that of the A. aerogenes extract. Both extracts were dialyzed and lyophilized for the same length of time. The ability of each crude enzyme extract to remove diacetyl from an aqueous solution at pH 7.2 and at 30°C is shown in Table 16. The reactions were carried out for 80 minutes with and without the addition of NADH and measured using the modified Owades and Jakovac method. Without NADH, yeast crude enzyme extract removed about five percent of the initial diacetyl concentration while the bacterial extract removed almost 20 percent of the starting diacetyl concentration. In the presence of NADH, better than 50 percent of the diacetyl was destroyed by the

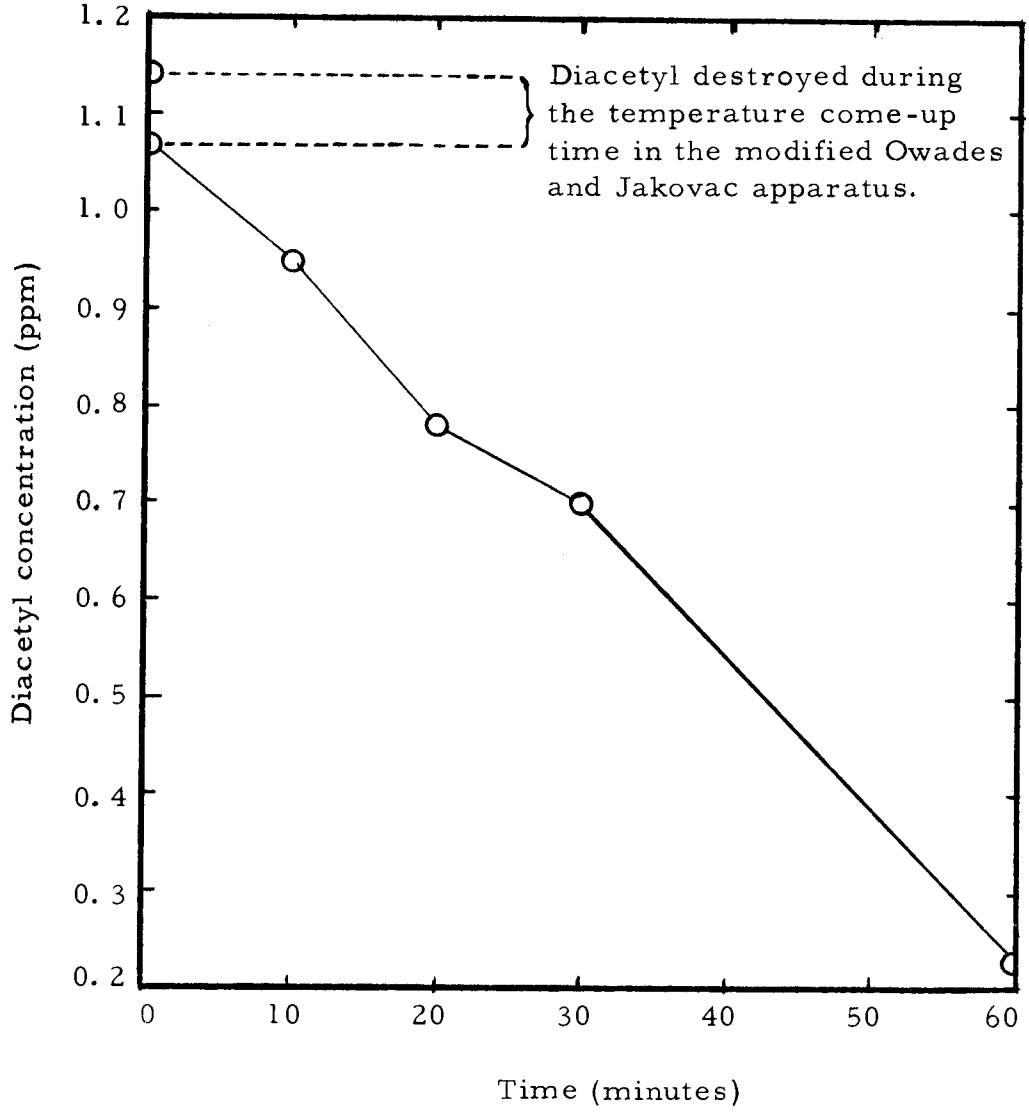

Figure 9. Ability of undialyzed crude enzyme extract (42. 5 mg) from Fleischmann's yeast to remove diacetyl from beer at a pH of 5. 25 and a temperature of 30° C reacting for the times indicated.

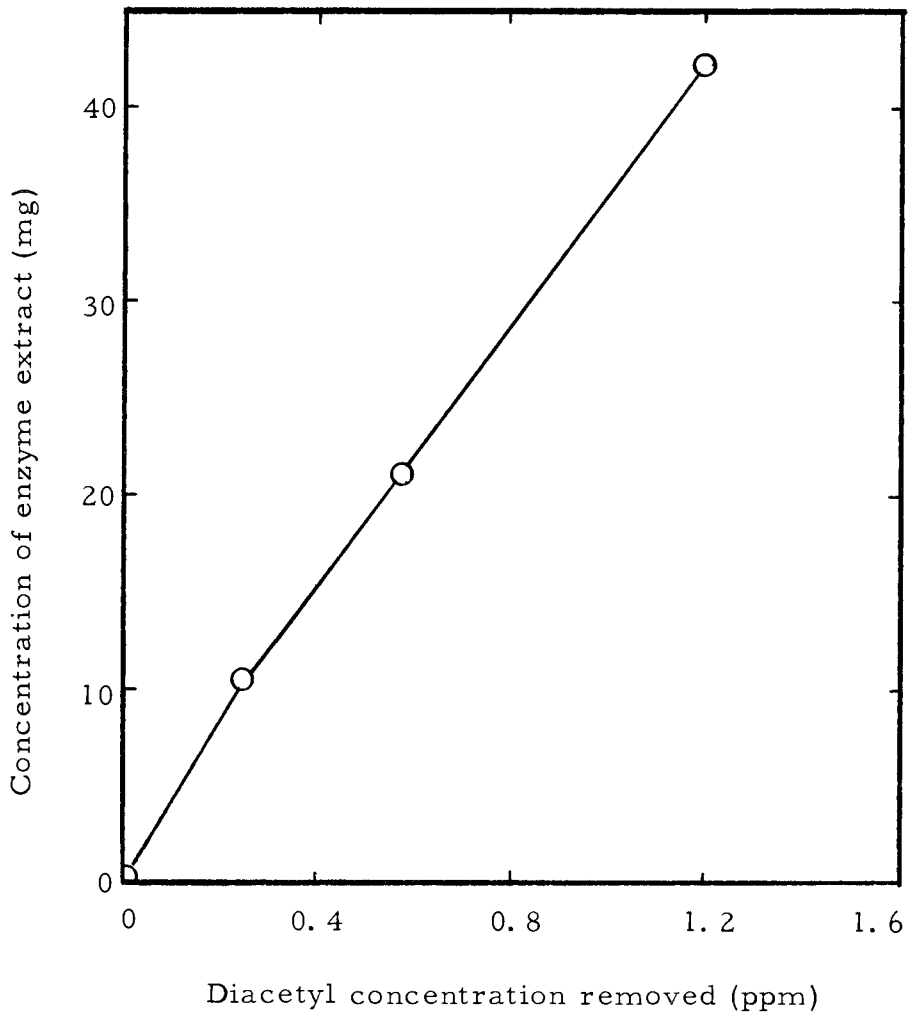

Figure 10. Ability of undialyzed crude enzyme extract from Fleischmann's yeast to remove diacetyl from an aqueous solution at pH 7. 2 in 45 minutes at a temperature of 30° C.

extract of the yeast, and the bacterial extract destroyed 100 percent of the initial diacetyl concentration.

Table 16. Ability of 50 mg of dialyzed crude enzyme extracts from A. aerogenes 8724 and Fleischmann's yeast to remove diacetyl from an aqueous solution at pH 7.2 with and without NADH at 30°C and for 80 minutes as determined using the modified Owades and Jakovac method

System[a]	Diacetyl concentration (ppm)		
	No enzyme present	Fleischmann's yeast crude extract[b]	A. aerogenes 8724 crude extract[c]
Buffer + diacetyl[d]	2.05	1.95	1.65
Buffer + diacetyl + NADH[e]	2.06	1.00	0

[a]Sufficient 0.1 M potassium phosphate buffer was added in each case to bring the final volume to 20 ml.

[b]Cells were disrupted using the Eaton press and were then centrifuged, dialyzed and lyophilized.

[c]Cells were disrupted by sonication and were then centrifuged, dialyzed and lyophilized.

[d]The same initial concentration of diacetyl was employed for each sample.

[e]A NADH concentration of 5 mg/ml was used.

A. aerogenes 8724 was not the only strain of A. aerogenes capable of destroying diacetyl. Crude enzyme extracts (diacetyl reductase) from four A. aerogenes strains were prepared and assayed for their diacetyl reductase content. Strain 8724 was found to contain far less diacetyl reductase activity than any of the other

strains tested, as shown in Table 17. It was noticed that upon prolonged dialysis (48 hours instead of 24 hours) the crude enzyme preparations took on a very turbid appearance. In Table 17, for each extract tested, the first sample utilized the turbid crude extract preparation while the extract used for the second sample was first centrifuged to clarify it. The clear supernatant liquid was then subsequently tested for diacetyl reductase activity.

Experiments were carried out on diacetyl reductase from A. aerogenes 8724 to verify results obtained by Pack (49). It was found that EDTA dialysis did not effect the enzyme's activity. Coupling of the enzyme system with alcohol dehydrogenase was accomplished, and NADH concentration was maintained at a constant level in the system. Also, attempts to reverse the activity of diacetyl reductase were not successful.

Figure 11 shows the effect of different concentrations of alcohol on the ability of diacetyl reductase to remove diacetyl from an aqueous solution when assayed under the previously described conditions with a continuous recording spectrophotometer. A concentration of 3. 3 percent alcohol inhibited the enzyme approximately 42 percent. A ten percent solution of alcohol inhibited about 69 percent of the activity while a 16. 7 percent alcohol solution inhibited 80 percent of the enzyme's original activity.

In all of the cell-free crude enzyme extracts tested, an

Table 17. Comparison of A. aerogenes strains for diacetyl reductase activity

Strain	Protein concentration (mg/ml)	Total activity (units/ml)	Specific activity (units/mg protein)
A. aerogenes 8308[a]	6.50	64,500	9,900
A. aerogenes 8308[b]	4.35	47,600	10,900
A. aerogenes 8724[a]	4.70	27,800	5,900
A. aerogenes 8724[b]	4.15	27,800	6,700
A. aerogenes 12658[a]	6.70	62,500	9,300
A. aerogenes 12658[b]	5.65	66,700	11,800
A. aerogenes O. S. U.[a]	6.30	66,700	10,600
A. aerogenes O. S. U.[b]	4.80	52,600	11,000

[a] The crude extract was prepared in the usual manner except that dialysis lasted 48 hours instead of 24 hours. After dialysis the extract appeared turbid.

[b] The crude extract was prepared as above; however, the turbid extract was recentrifuged for another one and one-half hours at 27,750 X g. The clear supernatant liquid was then subsequently tested for diacetyl reductase activity.

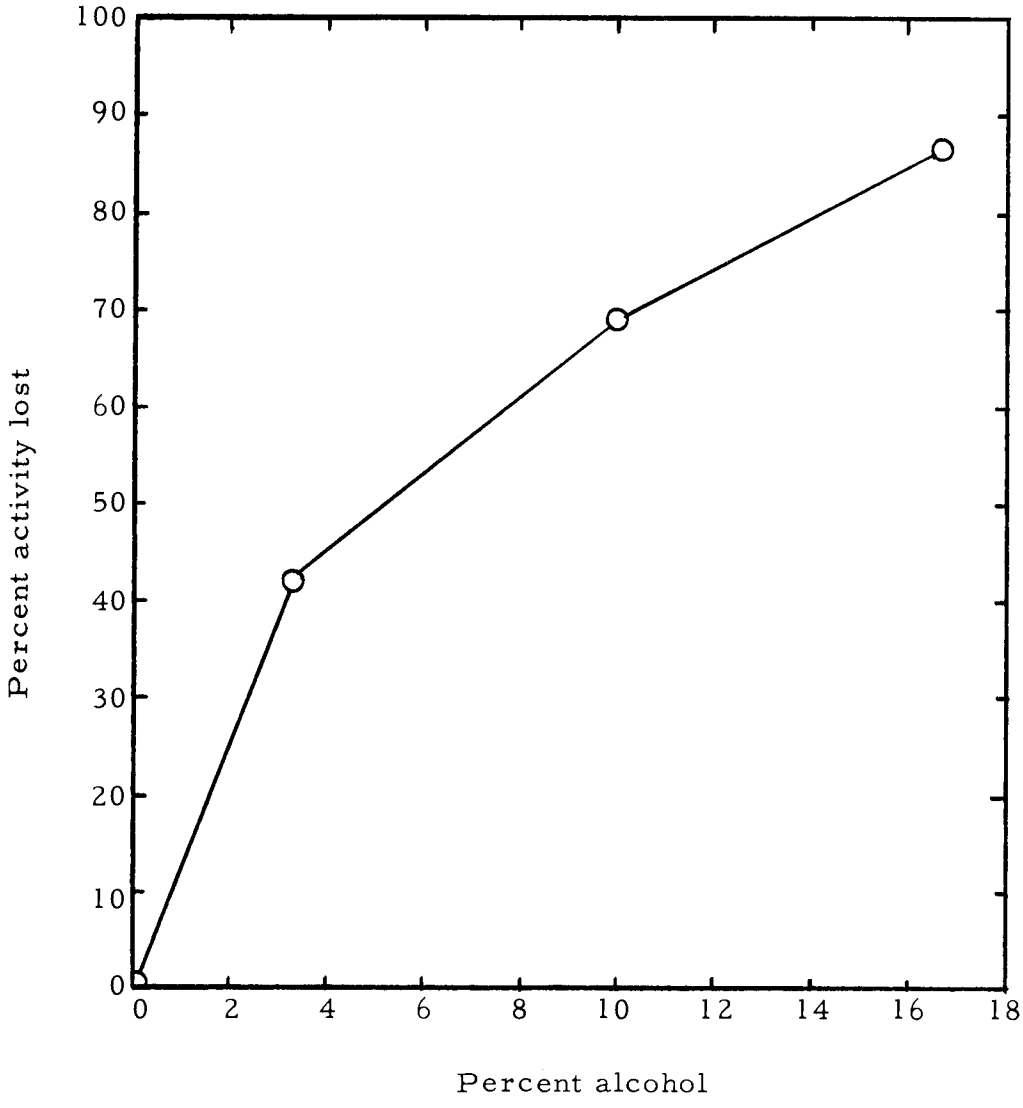

Figure 11. Effect of different concentrations of alcohol on the ability of diacetyl reductase from A. aerogenes to remove diacetyl when assayed with a continuous recording spectrophotometer.

endogenous level of NADH oxidation was apparent, even in the absence of the substrate diacetyl. Juni and Heym (36) referred to this endogenous activity as NADH oxidase. Even though their enzyme preparations were purified about 15 fold, they still had to subtract the NADH oxidase activity from the absorbance change observed when a substrate for the enzyme under question was added. For this study attempts were made to separate the NADH oxidase activity from the diacetyl reductase activity. Thermal denaturation and ammonium sulfate fractionation experiments were found to inactivate diacetyl reductase before NADH oxidase. Disc electrophoresis experiments showed at least three sites of NADH oxidation on the polyacrylamide gels; however, when the same experiment was tried in the presence of diacetyl, no new bands of NADH oxidation were observed. Later it was noticed Juni and Heym (35) had reported that diacetyl solutions should not be used in the presence of Tris buffer (tris-(hydroxymethyl)-aminomethane) because the buffer reacts rapidly with diacetyl.

Sephadex gel filtration was found to be highly successful at separating NADH oxidase and diacetyl reductase activities. Figure 12 shows that the first 20 fractions contained most of the NADH oxidase activity while fractions 20 through 40 were almost entirely free of NADH oxidase activity and were particularly high in their diacetyl reductase content.

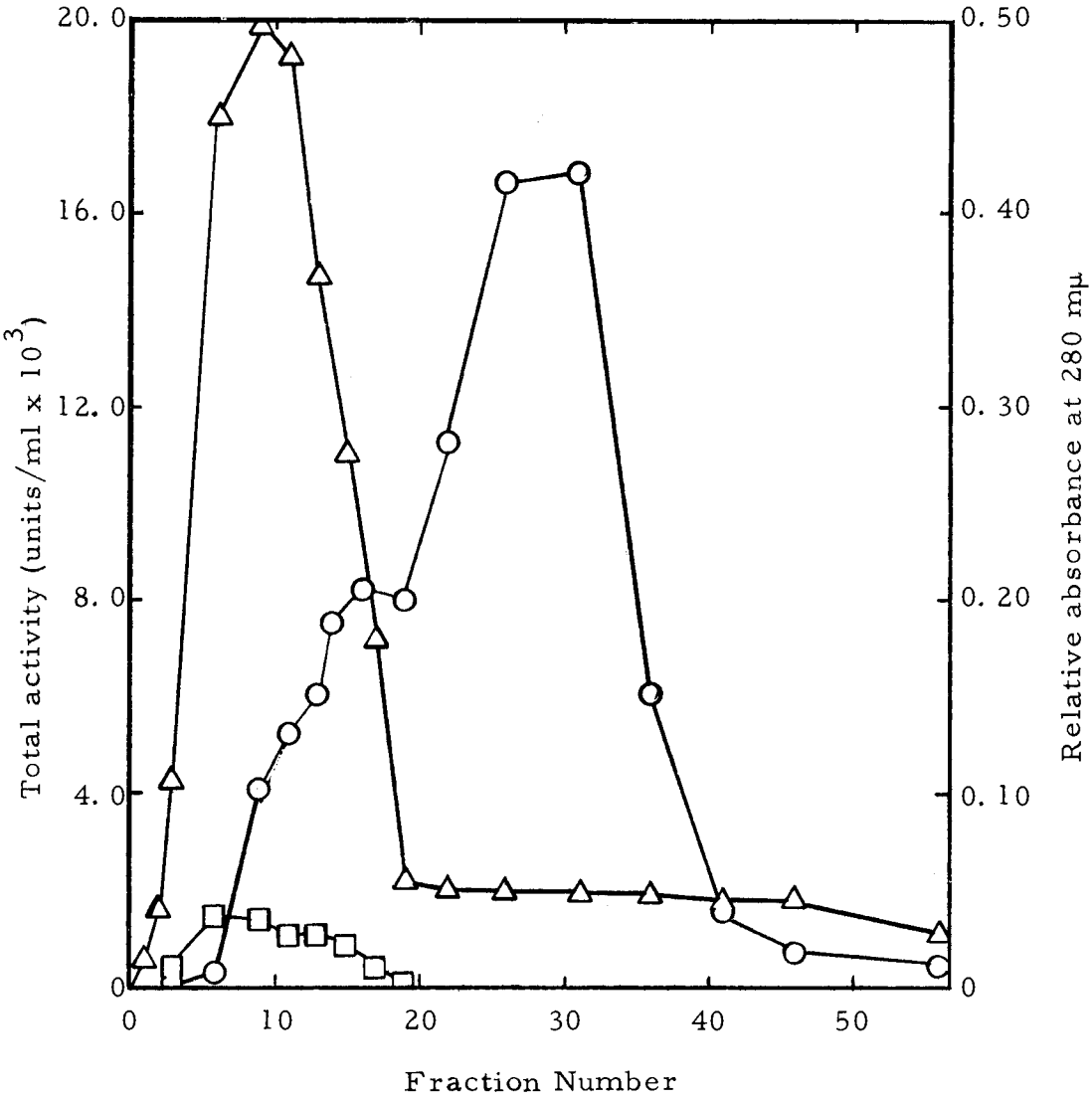

Figure 12. Sephadex elution pattern showing total activity of NADH oxidase (-□-), total activity of diacetyl reductase (-○-), and relative absorbance (-△-) of each 1. 35 ml fraction eluted from a 2. 5 by 36. 0 cm column of G-200 Sephadex.

Juni and Heym (36) showed that alcohol dehydrogenase was capable of reducing diacetyl at a limited rate. An attempt was made to determine whether lactate dehydrogenase might also have this ability. When lactate dehydrogenase was substituted in the place of diacetyl reductase in the spectrophotometric assay, no diacetyl was reduced.

A survey was conducted of several microorganisms for diacetyl reductase activity as determined spectrophotometrically. Table 18 lists the microorganisms tested, their growth medium, and whether or not NADH oxidase and diacetyl reductase activities were found. All of the microorganisms tested were found to have NADH oxidase activity while only A. aerogenes, and S. diacetilactis were found to have diacetyl reductase activity.

Non-enzymatic means of destroying diacetyl by incubating either tryptone, casamino acids, histamine, glutamine, or lysine in the presence of diacetyl for differing lengths of time resulted in no loss of diacetyl.

Table 18. Survey of microorganisms for diacetyl reductase activity[a]

Microorganism	Growth medium	NADH oxidase activity	Diacetyl reductase activity
1) "C"-mold	Citrate broth	+	-
2) S. cerevisiae var. ellipsoideus	Citrate broth	+	-
3) S. diacetilactis 18-16	Citrate broth	+	+
4) "R"-mold[b]	Citrate broth	+	-
5) Bakers' yeast (Red Star)[c]	---	+	-
6) Bakers' yeast (Fleischmann's)[d]	---	+	(-)[f]
7) A. aerogenes ATCC 8724	Citrate broth	+	+
	Glucose broth	+	+
8) S. faecalis O. S. U.[e]	Citrate broth	+	(-)[f]
	Glucose broth	+	(-)[f]
9) P. soyae ATCC 13621	Citrate broth	+	-
10) P. cerevisiae ATCC 10791	Citrate broth	+	-
11) A. melanogenus ATCC 9937	YCM	+	-
12) S. cerevisiae 2091	YCM	+	-
13) " " 2000-3	YCM	+	-
14) " " I	YCM	+	-
15) " " 2094-N	Citrate broth	+	-
16) " " 1538	Citrate broth	+	-

Table 18. Continued

Microorganism	Growth medium	NADH oxidase activity	Diacetyl reductase activity
17) S. cerevisiae 2094-P	YCM	+	-
18) " " PH3	Citrate broth	+	-
19) " " 2000	YCM	+	-
20) " " T	YCM	+	-
21) " carlsbergensis	Commercial wort	+	(-)[f]

[a]All extracts were dialyzed and lyophilized unless otherwise indicated. Diacetyl reductase activity was determined by spectrophotometric means by measuring the rate of oxidation of NADH at 340 mμ.

[b]This mold is thought to be a producer of high levels of riboflavin.

[c]The starch filler was not first removed by washing. It was then suspended in one percent glucose and aerated at room temperature for one hour before it was sonified.

[d]The bakers' yeast was ground with a mortar and pestle.

[e]These extracts were not lyophilized or dialyzed.

[f]When the activity was measured with the Owades and Jakovac apparatus instead of with the Gilford, the extracts were found to have activity.

DISCUSSION

Occurrence of Diacetyl in Beer

The modified Owades and Jakovac method was found to be quite reliable for determinations involving low levels of diacetyl in beer. It should be cautioned, however, that Vincent S. Bavisotto (4) noted that several breweries are having difficulties with the method. This points to the importance of proper techniques and careful handling of the apparatus. The author noted several pitfalls while working with the method. First of all, the alkaline tartrate reagent, when not refrigerated, frequently led to erroneous results. Also, the neoprene stoppers on the apparatus itself had to be replaced periodically due to excessive wear. The water level of the 65°C water bath could not be allowed to get too high or excess vaporization in the sample tubes resulted and the condensation in the inverted U-tubes eventually led to cloudiness in the hydroxylamine trapping solution. For the first 20 minutes of the determination, the gas flow had to be maintained at a rate much lower than originally described in the modified method. After this time, the flow rate was increased back to normal. For increased sensitivity in the low ranges of diacetyl concentration, it was found that a more sensitive spectrophotometer, such as the Beckman DU, should be used.

The author was surprised to see the low levels of diacetyl found during the survey of alcoholic beverages. This suggests that the diacetyl problem for breweries is sporadic in nature; diacetyl off-flavor is generally not a problem but when this problem does arise, its existence can be quite costly to the brewery.

The low diacetyl content of malt beverages in contrast to the much higher diacetyl content of wines and champagnes may be related to the initial protein content of these products. With wines, the yeasts have to synthesize a major part of their own amino acids, while in malt beverages, many of their requirements are satisfied by the constituents of the wort. If Owades' proposed mechanism of valine synthesis (Figure 2) is the major pathway of diacetyl synthesis, it would be clear, then, that under conditions where amino acids had to be produced by the cell in great quantities, much more diacetyl could result.

The organoleptic tests run concomitantly with the modified Owades and Jakovac determinations demonstrated that only in a few instances was the panel able to detect the relative level of diacetyl present in the samples.

Brewing Techniques and Procedures

As previously mentioned and as shown in the results (Figures 4 and 5), diacetyl production and destruction curves are typical of

all brewers' yeast fermentations. Yeast strain, yeast propagation methods, fermentation conditions, and composition of the wort all appear to be of great importance concerning diacetyl production. However, it has been said that other factors affecting the production of diacetyl are of secondary importance to the choice of yeast strain selected for the fermentation (53).

Several workers reported that various yeast strains produced differing levels of diacetyl (17, 37, 48), but none of these reports compared diacetyl production on a per cell basis. To make the results more meaningful, the relative diacetyl production per cell was used as the basis of comparison (Table 12). It is then clearly seen that one strain produced nearly 2.5 times as much diacetyl as the strain producing the least diacetyl. Differences as great as this are quite significant and cannot be disregarded as differences due only to fermentation variations. Owades et al. (48) suggested one possible reason for this variation when they noted that yeasts differed significantly in their ability to absorb valine from wort. It seems likely that research will be continued in this laboratory on yeast strain variations with respect to diacetyl production.

The addition of corn steep liquor to wort affected diacetyl production in a manner opposite to what was expected. It was proposed that its addition, as a minor constituent to wort, might possibly be a reason for the low diacetyl incidence of some beers. As shown on

Table 6, corn steep liquor is actually a very complex medium. The valine content of its relatively high amino acid fraction should have served as a feedback inhibitor of steps leading to diacetyl formation. Lactic acid, another major constituent of corn steep liquor, in its conversion to pyruvic acid results in the formation of NADH. In theory, the more NADH present, the less one would expect diacetyl formation to occur. Also, the pyruvate produced has a tendency to suppress diacetyl formation (21). So, it can be seen that even if the inositol content of the liquor had no effect on diacetyl formation, the other constituents discussed should have been sufficient to suppress diacetyl formation. It was found, however, that both the inositol and the corn steep liquor addition to the wort resulted in an increase in the amount of diacetyl produced. However, the increase resulting from the inositol addition does not correlate with the increase caused by the corn steep liquor addition. Therefore, the author has no explanation for the resulting increase in diacetyl as described above.

Studies on Diacetyl Removal

As mentioned previously, several groups have reported on yeast's ability to remove diacetyl from beer. Burger et al. (9) reported that both bakers' yeast and brewers' yeast were capable of removing diacetyl from beer. They reported that heat treated yeast cells were not capable of removing diacetyl. Table 13 confirms

their results. Table 13 also shows that yeast cells are not alone in their ability to remove diacetyl, but that apparently some bacterial cells also have this capacity. S. diacetilactis 18-16 removed diacetyl at a rate almost equal to that of yeast cells. This observation was not too surprising since it had already been established that crude enzyme extracts obtained from some bacteria were capable of destroying diacetyl.

The diacetyl destroying ability of whole yeast cells was made use of in experiments designed to remove diacetyl off-flavor from beer without imparting yeast autolysis off-flavor in its place. Even though yeast cells contained in dialysis tubing were capable of removing diacetyl from beer, serious yeast autolysis off-flavors resulted from their presence due to the lengthy exposure time required for the removal of diacetyl. However, one application of the use of live,whole yeast cells for diacetyl removal was entirely successful. Yeast cells, when impregnated in a diatomaceous earth filter bed, were found to be capable of destroying all the diacetyl from solutions percolated through the bed. This laboratory did not have the necessary equipment to continue studies dealing with maximum flow rates possible through such a filter. Also, it was not possible to continue studies on optimum yeast cell-diatomaceous earth ratios for such a filter.

The filtration system just described offers several advantages

to breweries plagued with sporadic diacetyl outbreaks. First, it is important to note that beer is filtered twice after fermentation in most brewery operations. It flows through a diatomaceous earth filter as it leaves the aging tank and enters the finishing tank, and likewise as it is pumped from the finishing tank to the holding tank (Figure 1). Yeast cells could be incorporated in the diatomaceous earth at the first filtration step if so desired. Second, yeast cells recovered from the fermenter have been shown to be entirely satisfactory for use in the filtration process. Third, the filtration process would not slow down production providing the time in the aging tank was cut down slightly. And fourth, the prolonged exposure of yeast cells to the beer, which is prevalent when breweries practice kräusening, would be avoided so that yeast autolysis problems would not occur. Due to the difference in settling rates (diatomaceous earth settles more quickly than yeast cells), if two filtration beds were used alternately, while one bed was filtering beer the other bed could be fluidized, the old yeast washed away, and fresh yeast impregnated into the bed. This practice would aid in preventing yeast autolysis off-flavor from occurring.

The Eaton press proved to be an efficient method for the preparation of crude enzyme extracts from yeast cells. Crude enzyme extracts of yeasts prepared in this manner, when undialyzed, were found to destroy diacetyl at a rapid rate. When the pH of the

medium was lowered to 4.9 or lower, much of the protein was denatured causing precipitation of the extract to occur. After exposure of several days, some diacetyl was destroyed, but the pH denaturation slowed the enzyme's activity to a rate too slow to make enzyme extract use competitive with that of whole yeast cell diacetyl removal. Dialyzed crude enzyme from yeast was found to destroy diacetyl in a manner quite similar to that of diacetyl reductase obtained from A. aerogenes. The enzymes from both organisms were able to destroy some diacetyl without NADH addition. Also, with each enzyme diacetyl reduction was greatly stimulated by the addition of NADH. The endogenous diacetyl destroying activity observed in each extract was probably the result of a low percentage of the original extract's residual NADH which had not been destroyed by the prolonged dialysis (48 hours at 5°C in several changes of distilled water).

Juni and Heym (36) described an active 2, 3-butanediol dehydrogenase system which they found to be present in yeasts and bacteria. This enzyme was also capable of oxidizing NADH in the presence of diacetyl. In all probability, the enzymes of the 2, 3-butanediol cycle described previously (Figure 3) are the same enzymes involved in this study. Juni and Heym suggested that several of the enzymes described in their proposed cycle were probably just the same enzyme performing more than one function.

It is certain that Green's diacetyl mutase (27) and Dolin's

pyruvic acid oxidase (15) are not similar to the enzymes involved in this study. Their enzymes required the addition of TPP and magnesium ion. Also, their enzymes were not stimulated by NADH addition and were inactivated by dialysis and lyophilization, whereas the enzymes described in this study were different with respect to each item just described.

Acetoin is formed primarily by two reaction mechanisms in yeast (16). In the first mechanism, the synthesis of acetoin is actually a side reaction of the alcohol fermentation. It involves a condensation of free acetaldehyde with "active acetaldehyde". The second reaction mechanism involves the condensation of pyruvic acid with "active acetaldehyde" to form alpha-acetolactic acid, an intermediary product in the synthesis of valine (Figure 2). Portno's observations (53) that the addition of valine to wort merely postpones diacetyl formation could be explained by the two acetoin pathways just described; early suppression of the valine synthesis pathway may block the second mechanism described above for the entire fermentation. However, as long as the yeast is able to use the first mechanism too, as soon as the anaerobic stage of fermentation begins the yeast will be able to produce diacetyl as a side reaction as described previously. The acetoin resulting from each of these pathways is converted to diacetyl, possibly in the presence of acetoin dehydrogenase and in the presence of NAD (16). The fact that

Drews et al. (16, 17) were able to reverse the diacetyl to acetoin reaction is of particular interest to this study because attempts to reverse the action of the enzyme diacetyl reductase were not successful as shown in this study and also previously reported by others (4, 36, 49, 58, 59, 66).

The 42 percent inhibition of diacetyl reductase activity resulting from a 3. 3 percent alcohol solution provides one more reason why diacetyl reductase is not suitable for use in beer where the alcohol content is generally about 3. 6 percent. Pack (49) had already stated that beer's low pH presented a seemingly unsurmountable problem with respect to the use of diacetyl reductase. The survey of microorganisms failed to provide an organism with a diacetyl reductase more suitable for use in beer than the one obtained from A. aerogenes. It is apparent that A. aerogenes 8724 is not necessarily the most active source of diacetyl reductase. Three other strains were tested that were all more active than strain 8724.

It was noticed that all the microorganisms surveyed were found to have some form of NADH oxidase. Gel electrophoresis results indicated that associated with the crude diacetyl reductase from A. aerogenes were at least three different types of NADH oxidase as shown by the presence of three distinct bands of NADH oxidation. Even Juni and Heym's 15 fold purification of diacetyl reductase did not get rid of the NADH oxidase activity (36). Of the methods tried

for this study, Sephadex gel filtration was found to be highly successful at separating diacetyl reductase from NADH oxidase. Since the intent of the experiment was not the mere protein purification of the enzyme, the actual protein purification achieved was not determined. It is suggested that in possible future experiments, protamine sulfate nucleic acid precipitation be employed before samples are run on the Sephadex column. Such a practice would prevent the masking of the 280 mμ protein absorbance by nucleic acids also absorbing at that wavelength; thus the protein concentration of the fraction could be more easily followed.

SUMMARY

The results obtained from studies on control of diacetyl off-flavor in beer may be summarized as follows:

1. Refinements in the modified Owades and Jakovac method of diacetyl determination were presented.

2. The diacetyl content of the alcoholic beverages surveyed was found to be much lower than expected, indicating that the diacetyl off-flavor problem is sporadic in its occurrence.

3. Compared on the basis of diacetyl produced per cell, a 2.5-fold difference was found to exist between yeast strains in their ability to produce diacetyl.

4. The addition of corn steep liquor to wort resulted in increased diacetyl production during the subsequent fermentation.

5. The addition of live, whole cells of S. diacetilactis 18-16 to solutions containing diacetyl resulted in diacetyl removal at a rate almost equal to that achieved by the addition of live, whole yeasts to the same solution.

6. Yeast cells impregnated in a diatomaceous earth filter bed are capable of destroying all of the diacetyl from solutions percolated through the bed.

7. Diacetyl removal from beer at its normal pH of 4.30 is accomplished at only a very slow rate when undialyzed crude enzyme

extract from yeast is the enzyme used.

8. Dialyzed crude enzyme extract from yeast cells was found to destroy diacetyl in a manner quite similar to that of diacetyl reductase from A. aerogenes with both enzymes being stimulated markedly by the addition of NADH.

9. Concentrations of alcohol similar to that found in beer were found to be quite inhibitory to diacetyl reductase activity.

10. Four strains of A. aerogenes were tested for their specific diacetyl reductase activity; three were found to have considerably more activity than strain 8724, the organism used exclusively so far in published work on diacetyl removal from beer.

11. Gel electrophoresis results indicated that at least three different NADH oxidases are active in the crude extract of diacetyl reductase obtained from A. aerogenes.

12. Sephadex gel filtration was found to be an excellent method for separating NADH oxidase activity from diacetyl reductase activity.

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