Enzymatic Removal of Diacetyl from Beer

II. Further Studies on the Use of Diacetyl Reductase

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Diacetyl removal from beer was studied with whole cells and crude enzyme extracts of yeasts and bacteria. Cells of Streptococcus diacetilactis 18–16 destroyed diacetyl in solutions at a rate almost equal to that achieved by the addition of whole yeast cells. Yeast cells impregnated in a diatomaceous earth filter bed removed all diacetyl from solutions percolated through the bed. Undialyzed crude enzyme extracts from yeast cells removed diacetyl very slowly from beer at its normal pH (4.1); 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 and the yeast extracts were stimulated significantly by the addition of reduced nicotinamide adenine dinucleotide (NADH). Diacetyl reductase activity of four strains of A. aerogenes was compared; three of the strains produced enzyme with approximately twice the specific activity of the other strain (8724). Gel electrophoresis results indicated that at least three different NADH-oxidizing enzymes were present in crude extracts of diacetyl reductase. Sephadex-gel chromatography separated NADH oxidase from diacetyl reductase. It was also noted that ethyl alcohol concentrations approximately equivalent to those found in beer were quite inhibitory to diacetyl reductase.

Diacetyl in beer causes an off-flavor which has been described as “lactic-diacetyl, buttery, or sarcina-like” (28). In addition, diacetyl is considered to be an off-flavor in wines (10, 20) and in citrus juices (2, 16). Different causes for this flavor defect have been suggested. In 1903, Claussen (5) described the causative agent of diacetyl production in beer to be a bacterium belonging to the genus Pediococcus. Shimwell and Kirkpatrick (26) studied diacetyl formation in beer and concluded that the causative agent was not a member of the genus Pediococcus but was in the genus Streptococcus. More recently, Burger et al. (3) reported that yeast cells produced diacetyl as a by-product during fermentation of wort used in beer manufacture. They also claimed that Lactobacillus pastorianus, a common bacterial contaminant in beer during the lagering stage, produced diacetyl. Kato and Nishikawa (14) also claimed 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 described as Lactobacillus pastorianus, a common bacterial contaminant in beer during the lagering stage, produced diacetyl. Kato and Nishikawa (14) also claimed that “beer sarcina” (a term used synonymously with pediococci), brewers' yeast, and L. pastorianus all produced diacetyl in beer. Several lacto...
diacetyl reductase from this bacterium to remove diacetyl from beer has been made (1). The present research is an extension of this latter work and concerns the limitations of the enzyme to control this flavor defect in the brewing industry.

MATERIALS AND METHODS

Diacetyl determinations. The colorimetric assay for diacetyl described by Owades and Jakovac (17) and modified by Pack et al. (19) was used.

Cultures. Yeasts of the genus Saccharomyces and bacteria of the Pedicoccus, Acetobacter, Aerobacter, and Streptococcus genera used in 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 & Co., Inc. All cultures were maintained in yeast-complete-medium (YCM), citrate broth (CB), or on wort agar (WA). YCM (pH 7.0) contained the following ingredients in g/liter: glucose, 20.0; tryptone, 20.0; and yeast extract, 10.0. Wort broth (pH 4.8) contained the following ingredients in g/liter: malt extract (Difco), 15.0; peptone (Difco), 0.78; maltose, 12.75; dextrin, 2.75; glycerol, 2.35; dipotassium phosphate, 1.0; and ammonium chloride, 1.0. CB was prepared as described by Sandine et al. (22). WA was available from Difco. YCM agar was prepared by adding 15 g of agar per liter of medium.

Diacetyl production and destruction. Two procedures were used to follow the appearance and loss of diacetyl. The first involved the use of 2 gal of wort prepared by adding one 3-lb can of Blue Ribbon malt extract (Premier Malt Products, Inc., Milwaukee, Wis.) and 3 lb of sucrose to 5 gal of water. The wort was then inoculated with 0.25 oz of Fleischmann’s dry yeast. Frequent agitation was used to hasten the start of the fermentation which was allowed to proceed for 7 days at 14 to 16 C. The pH and amount of diacetyl in the fermenting wort was determined at specific times using 20-ml portions at each sampling.

For the second method, two 250-ml flasks of wort broth were inoculated with a 5% inoculum of actively growing Saccharomyces 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 as described above.

Diacetyl production by yeast strains. To examine variability between yeasts in their ability to produce diacetyl, eight brewers’ strains of S. cerevisiae were inoculated in duplicate into 20 ml of sterile wort broth in culture tubes (25 by 250 mm, Corning no. 9820). After incubation for 63 hr at 10 C, one sample was removed for counting the yeast cells; diacetyl was determined on the duplicate 20-ml portion.

Diacetyl removal by heat-inactivated and live cells. Whole cells of bacteria or yeasts were heat-inactivated by rapidly bringing a cell suspension to 98 C and then rapidly cooling in ice water to 25 C. Suspensions of live and heat-inactivated cells (0.25 g) were incubated in triplicate for a given length of time at 25 C in the presence of diacetyl (20 µg/ml) under the conditions shown in Table 2; reduced nicotinamide adenine dinucleotide (NADH) was omitted in experiments with whole cells.

Diacetyl removal using whole yeast cells in dialysis tubing. A heavy Fleischmann’s yeast cell suspension was washed several times with 0.1 M phosphate

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**TABLE 1. Yeast and bacterial cultures used**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Medium*</th>
<th>Organism</th>
<th>Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saccharomyces cerevisiae var ellipsoides</td>
<td>CB</td>
<td>Saccharomyces cerevisiae</td>
<td>WA</td>
</tr>
<tr>
<td>S. cerevisiae 2091</td>
<td>YCM</td>
<td>Pedicoccus cerevisiae</td>
<td>WA</td>
</tr>
<tr>
<td>2000-3</td>
<td>YCM</td>
<td>Acetobacter pasteurianus</td>
<td>WA</td>
</tr>
<tr>
<td>I</td>
<td>YCM</td>
<td>A. melano-genus 9937</td>
<td>WA</td>
</tr>
<tr>
<td>2094-N</td>
<td>CB</td>
<td>Streptococcus diacetylactis</td>
<td>CB</td>
</tr>
<tr>
<td>1538</td>
<td>YCM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2094-P</td>
<td>CB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PH3</td>
<td>YCM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td>YCM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>YCM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. carlsbergensis Bakers’ yeast cake</td>
<td>CW</td>
<td>Aerobacter aerogenes</td>
<td>CB</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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* Abbreviations: CB, citrate broth; YCM, yeast-complete-medium; CW, commercial wort, Blitz Weinhard Co.; WA, wort agar.

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**TABLE 2. Experimental design for the assay of cells and enzyme extracts for diacetyl reductase activity by the modified Owades and Jakovac apparatus**

<table>
<thead>
<tr>
<th>Component</th>
<th>Tube no.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1-3</td>
</tr>
<tr>
<td></td>
<td>ml</td>
</tr>
<tr>
<td>Buffer (0.1 M KH₂PO₄)</td>
<td>19</td>
</tr>
<tr>
<td>Enzyme (5 mg/ml)</td>
<td>1</td>
</tr>
<tr>
<td>Reduced nicotinamide adenine dinucleotide</td>
<td>1</td>
</tr>
<tr>
<td>Diacetyl (20 ppm)</td>
<td>1</td>
</tr>
</tbody>
</table>

* Beer was substituted for buffer in experiments so indicated in the Results section.

b Whole cells (0.25 g) were used in place of enzyme in experiments so indicated in the Results section.
buffer (pH 7.2) and then with beer (pH 4.3). Quantities (50 ml) of the suspension were placed in cellophane dialysis tubing which was then immersed in beer (1 liter) held at 3.6°C, previously spiked to contain 0.5 ppm of diacetyl Samples (20 ml) were taken daily up to 6 days and tested for diacetyl. In some cases, the samples were also tested organoleptically by members of a taste panel.

Diatomaceous earth—yeast cell filtrations. A glass column (5 by 60 cm) with a coarse-porosity fritted glass filter disc was used in experiments designed to study filtration of beer as a means of diacetyl removal. The column was packed with 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 (18 cm), the remainder was poured off the top of the column. A refrigerated Gilson fraction collector was set to collect 5 ml of effluent liquid per tube. A solution of diacetyl (0.5 ppm) was then passed through the column to determine the void volume; the diacetyl content of eluate fractions was determined by the modified method of Owades and Jakovac (17).

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’s yeast, 20 g of dry yeast granules was mixed with 200 g of diatomaceous earth in 2,000 ml of distilled water (or beer). Since, as determined by plate count, 1 g of dry yeast was equivalent (on a per-cell basis) to 2.5 g of wet-packed yeast, 50 g of the wet-packed brewers’ yeast was mixed with 200 g of diatomaceous earth to obtain equal ratios of the constituents. The filter bed was prepared as described above. A diacetyl solution (0.5 ppm) was then passed through the filter to determine the extent of diacetyl removal by the live yeast cells impregnated in the column.

Bacterial cell-free crude extract preparation. Bacteria were grown from a 1% inoculum in 2 to 40 liters of sterile medium for 24 hr at 30°C. CB was the medium most frequently used, but glucose broth (omitting sodium citrate) was also used.

After growth, the cells were harvested with the use of a continuous-flow attachment for the Sorvall RC-2 refrigerated centrifuge at 12,100 X g with a flow rate of 300 ml per min. The packed cells were then washed directly 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 buffer to a volume of 50 ml.

Crude enzyme extracts were prepared by disrupting the cells in a Raytheon 10 kc sonic oscillator for 20 min. Cell debris was removed by centrifugation at 27,750 X g for 1.5 hr in the refrigerated centrifuge. The supernatant fluid was dialyzed against three, 4-liter changes of distilled water, with each dialysis lasting 8 hr. The crude enzyme was then lyophilized and stored at -20°C until used. Protein determinations on the extract were done by the method of Lowry et al. (15).

Yeast cell-free crude extract preparation. Yeasts were grown from a 1% inoculum in 2-liter amounts of sterile CB or YCM broth for 24 hr at 30°C. In some cases, such as with Fleischmann’s yeast, the yeast was used as supplied commercially and not grown in CB or YCM.

After growth, the cells were harvested with the use of the large-capacity centrifuge head (GSA) of the Sorvall RC-2 refrigerated centrifuge at 4,080 X g for 10 min. The packed cells were recovered 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.

A 10-ml amount of the heavy yeast cell suspension was added to the cylinder well of an Eaton cell press (9) which had been prechilled to dry-ice temperature. The cylinder remained in contact with the dry ice for 15 min to freeze the suspension. The piston was placed in the cylinder and a pressure of 10,000 lb/inch² was applied by means of a hydraulic press. The frozen cells, extruded through a small orifice in the bottom of the cylinder, were collected in a metal centrifuge tube. This material was thawed and then centrifuged at 27,750 X g for 1.5 hr. The supernatant fluid, when not used immediately, was dialyzed, lyophilized, and stored at -20°C. Protein determinations on the extract were done by the method of Lowry et al. (15).

Assay of crude cell-free extracts for diacetyl reductase. Enzyme assays were carried out by two methods. The first method involved the use of either a Cary (model 11) or a Gilford (model 2000) continuous recording spectrophotometer to measure the activity of the crude enzyme extracts by following changes in the absorbancy at 340 nm caused by the oxidation of NADH during diacetyl reduction. The reactions were initiated by the addition of diacetyl to solutions containing enzyme, NADH, and buffer. After the blank was adjusted to zero, the absorbancy following the addition of NADH was recorded. The diacetyl solution was then added to the cuvette, and the reaction was allowed to proceed at 25°C. The time in seconds (T) required for 50% reduction of the initial absorbancy was used for the calculation of the enzyme units present (1).

The second method involved the use of the Owades and Jakovac (17) apparatus to measure colorimetrically the amount of diacetyl present. Table 2 shows the experimental design for the assay of crude enzyme extracts by this method. Tubes 1–3 were used to determine the initial diacetyl concentration. Tubes 4–6 were used to detect enzyme activity in the absence of the cofactor, NADH. Tubes 7–9 and 10–12 were used to measure the enzyme activity for two different enzyme concentrations in the presence of cofactor.

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

Sephadex chromatography. Thermal denaturation, ammonium sulfate fractionation, disc electrophoresis, and Sephadex chromatography were used to separate
diacetyl reductase from endogenous NADH oxidase activity found in cell-free crude extracts of *A. aerogenes* 8724. Only the latter was successful. A Sepha-
dex column (2.5 by 45 cm) was packed with type
G-200 gel 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; elution data were expressed as fraction
numbers after the void volume was eluted. A 4-ml
amount of a solution (40 mg per ml) of the extract
was added to the top of the column. The concentra-
tion of protein remaining in each of the 50-drop
(1.35 ml) fractions eluted from the column was
followed by absorbancy readings at 280 nm with a
Gilford model 2000 spectrophotometer. These frac-
tions were then assayed for NADH oxidase and
diacetyl reductase activity.

**RESULTS**

**Diacetyl production and destruction.** Figure 1
shows a diacetyl production and destruction curve

![Graph showing diacetyl production and pH vs. time](image)

**FIG. 1.** Diacetyl produced (△) and pH attained (■) at 14 to 16 C by Fleischmann’s yeast after different times of incubation in 2 gal of wort.

**TABLE 3.** Cell population and diacetyl produced by various strains of *Saccharomyces cerevisiae* after incubation at 10 C in wort broth for 63 hr

<table>
<thead>
<tr>
<th>Strain</th>
<th>Standard plate count/ml</th>
<th>Diacetyl produced</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Amt</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ppm</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>0.08</td>
</tr>
<tr>
<td>2000-3</td>
<td></td>
<td>0.08</td>
</tr>
<tr>
<td>2000</td>
<td>1.70 X 10⁷</td>
<td>0.54</td>
</tr>
<tr>
<td>1538</td>
<td>1.18 X 10⁷</td>
<td>0.59</td>
</tr>
<tr>
<td>PH3</td>
<td>1.73 X 10⁷</td>
<td>0.62</td>
</tr>
<tr>
<td>2091</td>
<td>8.00 X 10⁶</td>
<td>0.63</td>
</tr>
<tr>
<td>1</td>
<td>1.25 X 10⁷</td>
<td>0.70</td>
</tr>
<tr>
<td>T</td>
<td>1.17 X 10⁷</td>
<td>0.79</td>
</tr>
<tr>
<td>2094-N</td>
<td>1.83 X 10⁷</td>
<td>1.00</td>
</tr>
</tbody>
</table>

a Uninoculated wort broth.
b Strain 2000-3 grew poorly in this medium.
typical of the yeast fermentations at 14 to 16 C;
the pH of the wort indicates the extent of the
fermentation. Diacetyl production was maximum
at 48 hr and then decreased with time. In another
experiment conducted at 10 C, the diacetyl peak
occurred at 96 hr. One batch of beer prepared
from inoculated wort was bottled after the 6th
day of the fermentation. After 2 more weeks of
storage at 20 C, the yeasts settled to the bottom
of the bottles and the diacetyl was found to have
disappeared completely.

Table 3 shows the amount of diacetyl produced
by the eight strains of *S. cerevisiae*. It may be
seen that under these conditions, a 2.5-fold dif-
fERENCE in diacetyl production occurred between
the lowest (strain 2000) and highest (strain 2091)
diacetyl producers.

**Studies on diacetyl removal.** The effect of using
live, whole cells and heat-inactivated cells of
yeast and *S. diacetilactis* to remove diacetyl from
an aqueous buffered solution is shown in Table 4.
The use of Fleischmann’s yeast resulted in the
greatest diacetyl removal (90%). The mixture of
brewers’ yeast strains resulted in the removal of
75% of the diacetyl, whereas *S. diacetilactis* 18-16
destroyed 70% but in much less time. Heat-
inactivated cells of each of the above suspensions
were not capable of reducing any of the added
diacetyl.

Diacetyl was removed from beer by yeast cells
contained in a dialysis tubing (Fig. 2). Flavor-
panel results of one such experiment indicated
that extensive yeast autolysis had occurred during
a 3-day reaction time. Nevertheless, the diacetyl
level of the beer was decreased by this technique.

The diatomaceous earth filter beds also elimi-
nated all the diacetyl in the solutions tested, and
yeast autolysis was not a problem. Figure 3 com-

**TABLE 4.** Ability of live whole cells and heat-killed
cells to remove diacetyl (30 ppm) from a 20-ml
aqueous solution buffered at pH 7.2 at a
temperature of 25 C

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Cell conc (g/tube)</th>
<th>Reaction time (hr)</th>
<th>Heat-killed cells</th>
<th>Live cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brewers’ yeast*</td>
<td>0.24</td>
<td>275</td>
<td>0</td>
<td>75</td>
</tr>
<tr>
<td>Bakers’ yeast*</td>
<td>0.24</td>
<td>275</td>
<td>0</td>
<td>90</td>
</tr>
<tr>
<td><em>S. diacetilactis</em></td>
<td>18-16</td>
<td>0.27</td>
<td>3</td>
<td>70</td>
</tr>
</tbody>
</table>

* Cell weight was based on wet-packed cells.
S *Saccharomyces cerevisiae* 2000, I, and T were
mixed in equal amounts.

Fleischmann’s yeast.
Fig. 2. Ability of 50 ml of a heavy suspension of live whole yeast cells contained in dialysis tubing to remove diacetyl from beer (pH 4.3) at a temperature of 3.6 C.

Fig. 3. Comparison of the ability of diatomaceous earth (▲) and diatomaceous earth impregnated with yeast cells (□) to prevent the penetration of diacetyl through filter beds 18 cm deep and 5 cm in diameter.

Removal of diacetyl from beer was not encouraging. Table 5 shows results with extracts of Fleischmann's yeast; at pH 4.3 and at 5 C, 42 mg of extract was able to destroy only 9% of the diacetyl from a 1.1-ppm initial concentration in 64 hr. The low pH of the beer caused much of the crude enzyme extract to precipitate. At this pH with a reaction time of 2 hr at 30 C, no measurable diacetyl destruction was apparent. When (Fig. 4) the pH of the beer was raised with sodium hydroxide to 5.25, essentially the same concentration of extract removed more than 80% of the diacetyl at 30 C in 1 hr. It was also shown that as the concentration of the undialyzed crude

Diacetyl reductase activity of crude cell-free extracts. Initial attempts to demonstrate that crude enzyme extracts of yeasts were capable of removing diacetyl from beer were not encouraging. Table 5 shows results with extracts of Fleischmann's yeast; at pH 4.3 and at 5 C, 42 mg of extract was able to destroy only 9% of the diacetyl from a 1.1-ppm initial concentration in 64 hr. The low pH of the beer caused much of the crude enzyme extract to precipitate. At this pH with a reaction time of 2 hr at 30 C, no measurable diacetyl destruction was apparent. When (Fig. 4) the pH of the beer was raised with sodium hydroxide to 5.25, essentially the same concentration of extract removed more than 80% of the diacetyl at 30 C in 1 hr. It was also shown that as the concentration of the undialyzed crude

<table>
<thead>
<tr>
<th>Beer</th>
<th>Crude enzyme extract (mg/ml)</th>
<th>Reaction time (hr)</th>
<th>Temp</th>
<th>Initial diacetyl concn (ppm)</th>
<th>Per cent diacetyl removed</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>21</td>
<td>2</td>
<td>30 C</td>
<td>1.31</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>42</td>
<td>2</td>
<td>30 C</td>
<td>1.31</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>42</td>
<td>64</td>
<td>5 C</td>
<td>1.10</td>
<td>9</td>
</tr>
</tbody>
</table>

a Diacetyl was added to increase the concentration to the levels indicated.

b In each case, after a short time the low pH caused the enzyme extract to precipitate.

c Triplicate analyses were made.
enzyme extract from Fleischmann's yeast was increased, the amount of diacetyl removed in 45 min at pH 7.2 and at 30 C increased (Fig. 5). 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.

A comparison of the ability of crude enzyme extract of *A. aerogenes* 8724 and Fleischmann's yeast to remove diacetyl from an aqueous solution at pH 7.2 at 30 C is shown in Table 6. Without NADH, yeast crude enzyme extract removed about 5% of the diacetyl, whereas the bacterial extract removed almost 20%. In the presence of NADH, better than 50% of the diacetyl was destroyed by the extract of the yeast, whereas the bacterial extract reduced 100% of the diacetyl. Diacetyl reductase was not limited to strain 8724 of *A. aerogenes*; three other strains were also active (Table 7).

Figure 6 shows the effect of ethyl alcohol concentration on the ability of diacetyl reductase to remove diacetyl from an aqueous solution when assayed with the continuous recording spectrophotometer. A concentration of 3.3% alcohol inhibited the enzyme 42%; 10% alcohol inhibited 69%, and 16.7% alcohol inhibited 80% of the enzyme activity.

In all of the cell-free crude enzyme extracts tested, an endogenous level of NADH oxidation was apparent, even in the absence of the substrate diacetyl. Juni and Heym (13) referred to this

![Graph](http://aem.asm.org/content/27/6/654.full)

**Fig. 5.** Ability of undialyzed crude enzyme extract from Fleischmann's yeast to remove diacetyl from an aqueous solution at pH 7.2 in 45 min at a temperature of 30 C.

**Fig. 6.** Effect of alcohol concentration on the activity of diacetyl reductase from *A. aerogenes*.

<table>
<thead>
<tr>
<th>Table 6. Effect of enzyme extracts from <em>Aerobacter aerogenes</em> 8724 or Fleischmann's yeast on diacetyl present in pH 7.2 phosphate buffer after incubating for 80 min at 30 C.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>System</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Buffer + diacetyl</td>
</tr>
<tr>
<td>Buffer + diacetyl + reduced nicotinamide adenine dinucleotide</td>
</tr>
</tbody>
</table>

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

* Dialyzed, crude extract (50 mg) was used.

**Table 7. Comparison of *Aerobacter aerogenes* strains for diacetyl reductase activity.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Protein concn (mg/ml)</th>
<th>Total activity (units/ml)</th>
<th>Specific activity (units/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8308</td>
<td>6.50</td>
<td>64,500</td>
<td>9,900</td>
</tr>
<tr>
<td>8724</td>
<td>4.70</td>
<td>27,800</td>
<td>5,900</td>
</tr>
<tr>
<td>12658</td>
<td>6.70</td>
<td>62,500</td>
<td>9,300</td>
</tr>
<tr>
<td>OSU</td>
<td>6.30</td>
<td>66,700</td>
<td>10,600</td>
</tr>
</tbody>
</table>
endogenous activity as NADH oxidase. Even though their dehydrogenase enzyme preparation was partially purified, they still observed significant NADH oxidase activity. In the present study, attempts were made to separate the NADH oxidase from diacetyl reductase. Thermal denaturation and ammonium sulfate fractionation experiments were found to inactivate diacetyl reductase more readily than NADH oxidase. Disc electrophoresis experiments showed at least three sites of NADH oxidation on the polyacrylamide gels; however, no new bands of NADH oxidation were observed in the presence of diacetyl.

Sephadex chromatography was useful in separating NADH oxidase and diacetyl reductase activities. Figure 7 shows that the first 20 fractions contained most of the NADH oxidase activity, whereas fractions 20-40 were almost entirely free of the oxidase but were enriched for diacetyl reductase.

DISCUSSION

Diacetyl production and destruction (Fig. 1) are typical of all brewers' yeast fermentations. Strain, propagation methods, fermentation conditions, and composition of the wort all are important 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 (21).

Other workers have reported that yeast strains may produce different amounts of diacetyl (14, 18), but none of these reports related diacetyl production to the amount of growth. To make the results more meaningful, the relative diacetyl production per cell was used as the basis of comparison (Table 3); one strain produced nearly 2.5 times as much diacetyl as the strain producing the least diacetyl. Differences as great as this may be significant, especially when mild-flavored beer is being produced. Owades et al. (18) suggested one possible reason (feedback inhibition) for strain variation in diacetyl production when they noted that yeasts differed significantly in their ability to absorb valine from wort.

Burger et al. (4) reported that both bakers' yeast and brewers' yeast were capable of removing diacetyl from beer. They also noted that heat-treated yeast cells were not capable of removing diacetyl. These results were confirmed in the present study (Table 4). It also was shown that yeast cells are not alone in their ability to remove diacetyl, but that some bacterial cells also have this capacity; Streptococcus diacetilactis 18-16 removed diacetyl more rapidly than yeast cells.

The diacetyl-destroying ability of whole yeast cells was utilized in experiments designed to remove diacetyl off-flavor from beer. Even though yeast cells held in dialysis tubing were capable of removing diacetyl, yeast autolysis off-flavors resulted from the lengthy exposure time. However, one application of the use of live yeast cells for diacetyl removal was successful. Yeast cells, when impregnated in a diatomaceous earth filter bed, removed all the diacetyl from solutions percolated through the bed.

The possibility of treating beer by filtration to remove diacetyl is suggested by these results. Beer is filtered through diatomaceous earth twice after fermentation in most brewery operations, as it leaves the aging tank and when it is pumped from the finishing tank to the holding tank. Yeast cells could be incorporated in the diatomaceous earth at the first filtration step if so desired. Yeast cells recovered from the fermenter could be used in the filtration process. Also, the prolonged exposure of yeast cells to the beer, which is prevalent when breweries practice krausening, would be avoided so that yeast autolysis problems would not occur.

Due to the difference in settling rates (diatomaceous earth settling 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 could be washed away, and fresh yeast could be impregnated into the bed. This practice would aid in preventing yeast autolysis off-flavor from occurring.

Dialyzed crude enzyme from yeast was found...
to destroy diacetyl in a manner quite similar to that of diacetyl reductase obtained from \textit{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 residual NADH which had not been removed by dialysis.

Juni and Heym (13) described an active 2,3-butanediol dehydrogenase which they found in yeasts and bacteria. This enzyme was also capable of oxidizing NADH in the presence of diacetyl. Seitz et al. (25) also noted these activities in \textit{S. diacetylactis} and pointed out that whereas the 2,3-butanediol dehydrogenase was reversible, the diacetyl reductase was irreversible; this also was true for the enzymes of \textit{A. aerogenes} (24). Thus, it seems that these two activities are catalyzed by different enzymes; otherwise one might expect the diacetyl reductase to be reversible also. Juni and Heym (13) suggested these activities (diacetyl and acetoin reduction) were catalyzed by the same enzyme. Proof of either alternative is lacking, however, but is being studied in these laboratories.

It seems that diacetyl mutase described by Green et al. (11) and pyruvic acid oxidase studied by Dolin (7) are not the same as diacetyl reductase. These enzymes required the addition of thiamine pyrophosphate and magnesium ion, were not stimulated by NADH addition, and were inactivated by dialysis and lyophilization.

The 42\% inhibition of diacetyl reductase activity resulting from a 3.3\% ethyl alcohol solution provides one more reason why diacetyl reductase is not suitable for use in beer in which the alcohol content is generally about 3.6\%. The low pH of beer presents a problem with respect to the commercial use of diacetyl reductase. Clearly, some method of protecting the enzyme from hydrogen ions will be necessary, and such studies are in progress. A means of regenerating NADH also will be desirable.

Gel electrophoresis results indicated that at least three different NADH-oxidizing enzymes were associated with the crude diacetyl reductase from \textit{A. aerogenes}. These could be removed from diacetyl reductase by Sephadex chromatography, but even their presence in the crude extracts did not prevent the use of the enzyme to remove diacetyl from beer under the conditions of this study. However, use of the enzyme to remove diacetyl from beer under commercial conditions will require either the use of whole cells or some other system to protect the enzyme.

\textbf{LITERATURE CITED}


REMOVAL OF DIACETYL FROM BEER


