Enzymatic Removal of Diacetyl from Beer

III. Enzyme Protection and Regeneration of Cofactor

JANET W. THOMPSON, J. SHOVERS, W. E. SANDINE, and P. R. ELLIKER

Department of Microbiology, Oregon State University, Corvallis, Oregon 97331

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Use of diacetyl reductase, a reduced nicotinamide adenine dinucleotide (NADH)-requiring enzyme, to eliminate diacetyl off-flavor in beer was studied. The crude enzyme was extracted from Aerobacter aerogenes and partially purified by ammonium sulfate precipitation or Sephadex chromatography. In the semipurified state, the enzyme was inactivated by lyophilization; in a crude state, the lyophilized extract remained stable for at least 4 months at -20 C. A 50% reduction in specific activity within 5 min was observed when crude diacetyl reductase was suspended (5 mg of protein/ml) in phosphate buffer at pH 5.5 or below; a similar inactivation rate was observed when the crude enzyme was dissolved in a 5% aqueous ethyl alcohol solution. Effective crude enzyme activity in beer at a natural pH of 4.1 required protection of the enzyme in 10% gelatin. Incorporation of yeast cells with the gel-protected enzyme provided regeneration of NADH. Combinations of yeast, enzyme, and gelatin were tested to obtain data analyzed by regression analysis to determine the optimal concentration of each component of the system required to reduce the level of diacetyl in spiked (0.5 ppm) beer to less than 0.12 ppm within 48 hr at 5 C. The protected enzyme system was also effective in removing diacetyl from orange juice (pH 3.8) and some distilled liquors.

Diacetyl has long been considered a serious off-flavor component in beer (18). It has been the subject of several recent reports (3, 5, 6) and also has been reported as a growing problem in frozen orange juice (2, 8, 9, 10). Marketing demands for beer have shifted in favor of a mild-flavored product, making the diacetyl problem even more acute. Without the masking effect of stronger flavor components, diacetyl becomes increasingly noticeable and objectionable (13). Changes in the composition of wort, acceleration of production, and introduction of continuous fermentation have created conditions favorable to formation of diacetyl off-flavor.

Brewers have been only partially successful in controlling diacetyl levels in beer, usually at the expense of production efficiency. Extended lagering of the beer assures more complete diacetyl removal, but the longer holding periods and storage requirements make the process burdensome. The addition of fresh whole yeast cells to fermented beer (Kräusening) is another means of reducing the diacetyl content, but yeast autolysis may produce additional off-flavors in the finished product. Addition of valine to discourage diacetyl production by feedback inhibition also has been advocated (6, 13). Research carried out in the Department of Microbiology at Oregon State University has revealed the ability of several bacterial species to destroy diacetyl in milk cultures (4, 15, 16). By using the organism Aerobacter aerogenes 8724, Seitz et al. (16) demonstrated a diacetyl-destroying enzyme identified as diacetyl reductase. In a preliminary report, Bavisotto et al. (1) presented findings suggesting the possible commercial use of diacetyl reductase to control diacetyl off-flavor in beer. Subsequently, Tolls et al. (17) studied methods of using the enzyme in eliminating diacetyl from beer and pointed out limitations of the enzyme in flavor control. The present report further substantiates possible industrial use of the enzyme by providing a means to stabilize the enzyme in beer and to eliminate the use of substrate quantities of the expensive cofactor reduced nicotinamide adenine dinucleotide (NADH).

MATERIALS AND METHODS

Enzyme preparation. The organisms used as sources of enzyme were A. aerogenes 8724 and Streptococcus diacetilactis 18-16. Both are maintained in the culture
collection of the Department of Microbiology, Oregon State University; the A. aerogenes strain was originally obtained from the American Type Culture Collection, where the S. diacetilactis strain also has been deposited (ATCC no. 15346). Both organisms were maintained and grown by using citrate broth described by Sandine et al. (14).

Cells were grown in 8-liter quantities by using aerated carboys (nonaerated in the case of S. diacetilactis) or an aerated and pH-regulated New Brunswick microfermentor (capacity of 12 liters). Cells were harvested after 36 to 48 hr of growth with a Sorvall RC-2 refrigerated centrifuge operated at 5,000 × g for 15 min. The cells were then washed three times and resuspended in 0.1 M potassium phosphate buffer at pH 7.0. Cell suspensions (50-ml quantities with the consistency of light cream) were sonicated for 20 min in a Raytheon 10-kc sonic oscillator, and the cell debris was removed by centrifugation at 15,500 × g for 1.5 hr. The resulting supernatant fluid was dialyzed through three 2-liter distilled water changes over a 30-min period and then lyophilized or stored frozen as the crude, unpurified extract. Protein determinations for each extract were done by the method of Lowry et al. (7).

Crude extracts of diacetyl reductase were partially purified by using a column (2.5 by 45 cm) packed with G-200 Sephadex gel in 0.1 M potassium phosphate buffer at pH 7.0. Samples (4.0 ml) were added to the column, and 50-drop portions were collected every 5 min; 25 samples were assayed for enzyme activity. In other attempts at enzyme purification, protein precipitation by ammonium sulfate was carried out. The purified samples were further analyzed for activity maintenance after lyophilization and extended storage at room temperature, 5 C, and -20 C. Activity was also analyzed as a function of decreasing pH (7.0 to 3.0) and in a 5% aqueous ethyl alcohol.

Enzyme assays. The Gilford model 2000 continuous-recording spectrophotometer was used to measure enzyme activity recorded as the decrease in absorbancy of NADH at 340 nm. The total system (standard assay conditions) contained 2.7 ml of potassium phosphate buffer at pH 7.2, 0.1 ml of diacetyl reductase (5 mg/ml), 0.2 ml of NADH at a concentration of 2 mg/ml, and 0.1 ml of diacetyl at a concentration of 860 µg/ml. The resulting absorbancy decrease plots were converted to units of enzyme activity by two methods. The first used a definition of one unit of enzyme as the amount of enzyme which under standard assay conditions caused a 50% reduction of the absorbancy when 1/7, the reciprocal of time in seconds, equaled 10^* (1). The other means of determining enzyme activity was to record the change in absorbancy with time (chart speed, 2 inches/min), measured on the linear portion of the curve. Specific activity in each case was determined by calculating the units of enzyme present per milligram of enzyme extract tested.

Diacetyl determinations. Diacetyl was assayed by using the colorimetric method of Owades and Jakovac (11) as modified by Pack et al. (12).

Use of diacetyl reductase in beer. The enzyme system used for studies on diacetyl removal from beer consisted of gelatin (Swifts' Superclear G10) to protect the enzyme, yeast cells (Fleischmann's dry yeast, Standard Brands, Inc., New York) to regenerate NADH, and the crude lyophilized enzyme. The mixture was prepared by warming 10 g of gelatin in 50 ml of distilled water to 40 C, at which temperature it was held until the gelatin was dissolved. After cooling to just above room temperature, the pH of the gel was adjusted to 7.0 ± 0.1 with 1 M NaOH before addition of the yeast and enzyme. One gram of yeast suspension and 0.1 g of dissolved, lyophilized enzyme preparation were then added, mixed, and spread as a thin film on sheets of polyethylene. After 24 hr at room temperature (25 C), the gel dried to a translucent, flexible film that could then be peeled from the polyethylene, cut into pieces approximately 1 cm square, and stored at 5 C.

Beer samples were spiked to 0.5 ppm of diacetyl by the addition of diluted, pure diacetyl (K & K Laboratories, Jamaica, N.Y.). The gel-yeast-enzyme was added to the beer at the rate of 360 mg per 120 ml of beer; each 120 ml of beer sample contained 324.4 mg of gelatin, 32.4 mg of yeast, and 3.2 mg of enzyme. The beer samples were then incubated for periods of time ranging from 12 to 120 hr at 25 C and at 5 to 7 C. Final diacetyl concentrations after incubation were determined by using the method of Owades and Jakovac (11). Each component of the gel-yeast-enzyme system was prepared separately and in combination with one of the other components, and analyzed in beer to determine the role of each in the diacetyl removal system. The standards set for the satisfactory performance of the enzyme system in beer required that diacetyl be reduced from 0.5 to 0.1 ppm in a minimal amount of time or that it be reduced to 0.2 ppm or less within 48 hr. All tested combinations were incubated in beer at 5 to 7 C for 48, 72, and 96 hr and analyzed in duplicate during separate trial periods (four to six tubes per trial).

Procedures used to measure leakage of yeast and enzyme from the diacetyl removal system into beer included plate counts on potato dextrose agar and reuse experiments to show that elements of the gel-yeast-enzyme system remained intact and active through repeated uses in fresh-beer samples.

Use of diacetyl reductase in orange juice and distillers' products. The same conditions used to measure the ability of the enzyme system to remove diacetyl from beer were employed to test the effectiveness of diacetyl reductase in removing the compound from orange juice samples or distilled liquors. The orange juice was obtained commercially as the frozen concentrate; the liquor samples were supplied by Hiram Walker and Sons, Peoria, Ill.

RESULTS

Enzyme assays. Table 1 shows specific activity values of crude and partially purified enzyme fractions prepared from A. aerogenes. The apparent protective influence of protein and the inactivating effect of lyophilization may be noted. Also, less than two- and fourfold purification was obtained by ammonium sulfate and Sephadex chromatog-
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Table 1: Effect of freezing and lyophilization on specific activity of crude and partially purified fractions of diacetyl reductase

| Treatment     | Fraction | Crude extract | 50% AS precipitate | Sephadex eluate |
|---------------|----------|---------------|--------------------|-----------------|
| Unfrozen      |          | 86.5          |                    |                 |
| Frozen        |          | 85.6          | 117.6              | 285.2           |
| Lyophilized   |          | 81.6          | 25.2               | 4.0             |

a Results are expressed as units (change in absorbancy per minute) per milligram of enzyme protein. Assays were run on freshly prepared enzyme immediately after freezing or lyophilization.
b Ammonium sulfate.

diacetyl reductase in beer. Table 2 shows the effect of the individual components (gelatin, enzyme, or yeast) on the level of diacetyl remaining in beer (pH 4.1) after incubation at 5°C for 2, 4, and 9 days. Gelatin or enzyme alone were inactive, but yeast alone was active. Yeast and enzyme were no more active than yeast alone, suggesting the enzyme was not functional under these conditions, again because of the pH effect. Gelatin and enzyme also were inactive because there was no NADH for the enzyme. The complete system was the most active, reducing all the diacetyl in 4 days even at this low temperature.

The effect of varying the gelatin, yeast, and enzyme concentrations on the rate of diacetyl reduction in beer by using the protected enzyme system may be seen in Fig. 2-4, respectively. It is clear that the yeast cells are the most important variable, though the gelatin and enzyme concentrations also are important.

When these data were submitted to computer regression analysis, the equation and correlation coefficient shown in Table 5 resulted. From the equation, the optimal concentration of the three components was determined.

Use of diacetyl reductase in orange juice and distillers' products. Diacetyl analyses on distillers' samples along with proof data are given in Table 6. Successful reduction of diacetyl reductase was only measurable with the low-proof samples, since no color reaction occurred when diacetyl analyses were made on the three high-proof samples.

Table 2: Effect of storage temperature and time on specific activity of crude and partially purified fractions of diacetyl reductase

| Time (weeks) | Frozen crude extract | Lyophilized crude extract | 50% AS precipitate |
|--------------|----------------------|---------------------------|-------------------|
|              |                      | 22 C | 5 C | -20 C | 22 C | 5 C | -20 C | 22 C | 5 C | -20 C |
| 0            | 85.6                 | —    | —   | —    | 81.6 | —    | —    | 117.6 | —    | —    |
| 1            | 10.1                 | 75.5 | —   | —    | —    | —    | —    | 37.4  | 85.5 | —    |
| 2            | 0                    | 14.0 | —   | —    | —    | —    | —    | 0     | 16.0 | —    |
| 4            | 0                    | 15.0 | —   | —    | —    | —    | —    | 0     | 16.0 | —    |
| 8            | 0                    | 7.3  | —   | 24.2 | —    | —    | 76.6 | 0     | 9.3  | —    |
| 12           | 0                    | 3.4  | 79.0 | —    | —    | 76.6 | 0     | 4.0  | 109.6| —    |

a Results are expressed as in Table 1; —, indicates not determined.
b Frozen extracts were placed at the temperature indicated and held for the period shown before assay.
c Lyophilized enzyme was stored at 22, 5, and -20°C and assayed after the periods indicated.
d Ammonium sulfate.
FIG. 1. Activity of diacetyl reductase at pH levels between 7.0 and 4.2 and in the presence of 5% ethanol. Curves represent decrease in absorbancy as the result of NADH oxidation in the presence of diacetyl. Curves 1 to 5 are pH 7.0, pH 6.0, 5% ethanol, pH 5.1, and pH 4.3, respectively.

Table 3. Effect of gelatin concentration on the specific activity and rate of diacetyl reduction by diacetyl reductase system in phosphate buffer at different pH values

| Trial | Gelatin | pH | Specific activity | Removal time |
|-------|---------|----|------------------|--------------|
|       | g/liter |    |                  | min          |
| 1     | 15      | 7.0| 3,480            | 19           |
| 2     | 20      | 7.0| 3,280            | 20           |
| 3     | 15      | 4.1| 1,528            | 44           |
| 4     | 20      | 4.1| 2,186            | 31           |
| 5     | 25      | 4.1| -                | NR           |
| 6     | 15      | 7.0| 22,440           | 3            |
| 7     | 15      | 6.0| 20,240           | 3            |
| 8     | 15      | 5.1| 3,820            | 11           |
| 9     | 15      | 4.3| 1,114            | >60          |
| 10    | 15      | 7.0 | 5,600           | 8            |

a The complete system contained 5 mg of enzyme per ml prepared in the concentrations of gelatin indicated. Cuvettes contained 0.4 mg of NADH, and the reaction was started by adding 86 µg of diacetyl.

b Trials 6 through 10 employed a more active enzyme preparation than trials 1 through 5.

c Not determined.

d No reaction.

The initial concentration of diacetyl was 0.5 ppm, with the amount present at different times determined by the Owades and Jakovac method.

FIG. 2. Effect of gelatin concentration on rate of diacetyl reduction by diacetyl reductase system in beer at 5°C. Curves 1 to 4 are 2.5, 5.0, 10.0, and 15.0% gelatin, respectively.

In orange juice, the protected enzyme system functioned, but complete removal of diacetyl was not achieved (Fig. 5).

DISCUSSION

The results presented in Tables 1 and 2 on the stability of crude diacetyl reductase preparations from A. aerogenes emphasize that partially puri-
fied enzyme becomes much more labile to freezing and lyophilization. The crude extract, however, is quite stable to these treatments but, unfortunately, maintains activity for extended periods only when stored at -20 C. This imposes a storage inconvenience regarding commercial use of the enzyme. Whether this limitation will apply to the gelatin protected enzyme system will require further study. However, preliminary results have revealed that gel-yeast-enzyme mixtures stored at 25, 5, or -20 C lose no activity up to 4 months of storage.

Although initial experiments were complicated by flakes of gelatin-containing enzyme which made absorbancy readings somewhat inaccurate, the data (Table 3) suggested that gelatin would be a suitable protective agent for the enzyme in adverse pH and alcohol environments. Although this was true (Fig. 2), some method of regenerating cofactor for the enzyme was needed to avoid the necessity of adding substrate quantities of NADH. When it was noted that yeast cells alone and, more especially, when incorporated into gelatin were active in reducing diacetyl in beer (Table 4), it became apparent that yeasts were providing a constant source of reduced cofactor to allow the enzyme to function. This may occur through the coupling of the enzyme to alcohol or lactic dehydrogenase (1). Figure 3 emphasizes dramatically the dependency of diacetyl reductase on the presence of yeast cells when exogenous NADH is not provided. In fact, even in the absence of enzyme (Fig. 4), the yeast cells function quite well in reducing diacetyl since they contain diacetyl reductase (17) in addition to the NADH-regenerating system.

These findings suggest the possibility of using yeast cells immobilized in gelatin in the absence of added diacetyl reductase to control diacetyl off-flavors in fermented beverages. This could be done by using the strains of yeasts being used in the actual fermentation to minimize the possibility of contaminating beer with a foreign yeast. In this regard, the gelatin flasks prepared in this study, which contained enzyme and yeast cells, were tested for leakage of cells into beer. In three experiments in which 120 ml of beer was incubated at 5 C for 96 hr with 360 mg of the enzyme system, an average of 3 cells per ml were recovered; when an equivalent amount of yeast was similarly plated, 35,000 cells per ml were recovered. Thus, 99.991% of the yeasts are retained in the gelatin, even though the flakes were prepared by cutting the film. Preparation of the mixture in droplet form would probably prevent any leakage, and further work on this is in progress. Where it might be desirable to use yeast cells exclusively for diacetyl control, studies need to be made on conditions necessary in preparation of the cells to minimize cost and maximize the activity of the cells for diacetyl reductase and NADH regeneration.
In the brewing industry, a variety of chill-proofing (proteolytic) enzymes are currently used to enhance the clarity of the finished product. Usually, less than a pound of these agents is used per barrel of beer, and it was of interest to compare this customary rate of enzyme addition with that found necessary in the present study for the diacetyl reductase system. From the regression analysis (Table 5), the optimal concentration of the enzyme component was about 68 mg per 120 ml of beer; this is equivalent to 0.15 lb per barrel. Where the total weight of the enzyme system is concerned (490 mg/120 ml), this is equivalent to about 1.1 lb per barrel of beer. Although this may seem like a sizable quantity, the gelatin-yeast-enzyme system is recoverable from the beer after the diacetyl is reduced and does not actually represent an addition to the beer that might affect quality after processing. Furthermore, it was determined in this study that the gelatin-protected system was reusable after recovery from treated beer. In a typical experiment in which the initial use reduced the diacetyl in beer from 0.5 ppm to none in 96 hr, the first reuse left 0.23 ppm with complete removal by 192 hr. Even a third reuse of the recovered flakes lowered the diacetyl to 0.07 ppm after 192 hr.

### Table 5. Multiple regression equation for determining the optimal concentration of enzyme system components to reduce diacetyl in 120 ml of beer to less than 0.12 ppm within 48 hr at 5 °C

| Multiple regression equation | Multiple correlation coefficient (R²) | Optimal component concn (mg/120 ml of beer) |
|-----------------------------|-------------------------------------|---------------------------------------------|
| \( y = 0.6748 - 0.009787x_1 - 0.0000924x_1^2 - 0.032564x_2 + 0.001865x_2^2 - 0.000941x_3 + 0.000001858x_3^2 + 0.0005312x_1x_2 - 0.000001861x_1x_3 - 0.00002293x_2x_3 \) | 0.727 | Yeast \((x_1)\) | 67.74 | Enzyme \((x_2)\) | 1.67 | Gelatin \((x_3)\) | 420.44 |

### Table 6. Diacetyl content and proof of distillers' products

| Sample     | Proof | Diacetyl (ppm)* | Diacetyl remaining after 120 hr |
|------------|-------|-----------------|---------------------------------|
| F12-1      | 3.6   | 2.7             | 0                               |
| F12-2      | 115   | 1.1             | 0                               |
| F15-1      | 5.5   | 2.7             | 0.6                             |
| F15-2      | 116   | 1.1             | 0.6                             |
| Rye distillate | 138   | 5.1             | 0.6                             |

* Diacetyl values were furnished by supplier.

\( ^b \) No color reaction occurred.

Attempts to contain the gelatin flakes in gauze bags suspended in the beer to facilitate easy removal resulted in some inhibition of diacetyl removal; reduction of diacetyl from 0.5 to 0.23 ppm occurred in 96 hr, whereas control beer with the flasks evenly distributed throughout contained no diacetyl after this period. Thus, it would appear that the protected enzyme system should be free to mix with the beer for maximal diacetyl removal efficiency.

It is clear from the results of this study (Table 6 and Fig. 5) that further research is necessary to establish the applicability of diacetyl reductase to the removal of diacetyl from orange juice and...
distillers' products. The preliminary data would appear to justify further studies in this regard. The suitability of the protected enzyme system, however, to control this flavor defect in beer seems well established. Results on the application of the system on a commercial scale will be reported in the near future.

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