Diacetyl and Acetoin Production by *Lactobacillus casei*

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Agitation of broth cultures of *Lactobacillus casei* retarded cellular dry weight accumulation but enhanced production of both diacetyl and acetoin. Addition of pyruvate overcame this retardation, but addition of sulfhydryl-protecting reagents did not. Both pyruvate and citrate enhanced accumulated dry weight of *L. casei* incubated without agitation, but only pyruvate increased diacetyl accumulation. Both actively dividing cells and cells suspended in buffer converted pyruvate to diacetyl and acetoin. Maximum production of diacetyl and acetoin occurred during the late logarithmic or early stationary phases. Cells isolated from pyruvate- or citrate-containing cultures showed the greatest ability to convert pyruvate to diacetyl and acetoin. The optimum pH for diacetyl and acetoin formation by whole cells was in the range of 4.5 to 5.5. The presence of citrate or acetate enhanced diacetyl and acetoin formation by *L. casei* cells in buffer suspension.

*Lactobacillus casei*, an organism utilized in several food fermentations, produces diacetyl as one of its more important volatile constituents (1, 11, 15). Since diacetyl is important in the flavor of certain fermented foods, production and utilization of this compound can play a significant role in the development of flavor. We reported previously that citrate stimulates the growth of *L. casei* (4) and that citrate and pyruvate increase the levels of diacetyl reductase in this organism (3). During these studies, pyruvate and citrate were found to have a profound influence on diacetyl production (5). Thus the present investigation was initiated to elucidate the nature of certain chemical and environmental factors which can influence diacetyl and acetoin production by actively growing cultures and by isolated whole cells of *L. casei*.

**MATERIALS AND METHODS**

Organism and culturing conditions. *L. casei* 393, obtained from the American Type Culture Collection, was used throughout this study. The organism was propagated routinely by culturing in Elliker broth (Difco) for 36 to 48 hr at 30 C.

To determine the effects of citrate or pyruvate, given amounts of sodium citrate or sodium pyruvate were dissolved in deionized water and added to Elliker broth, which was then sterilized by autoclaving for 15 min at 121 C. Acetaldehyde and sodium acetate solutions were sterilized by autoclaving (2), and solutions of glutathione, 2-mercaptoethanol, and dithiothreitol were sterilized by membrane filtration (0.45-μm pore size; Millipore Corp., Bedford, Mass.) before addition to sterile Elliker broth. All cultures were incubated at 30 C. Aerobic conditions were maintained by agitating cultures in a gyratory shaker (model G-25, New Brunswick Scientific Co., New Brunswick, N.J.) which was operated at 90 strokes/min unless specified otherwise. Static conditions refer to undisturbed incubation. Cell growth was measured by determining absorbance at 660 nm and converting to dry weight of cells as described previously (4).

**Diacetyl and acetoin assay.** Diacetyl levels were determined by the specific colorimetric method of Pack et al. (13). The total diacetyl plus acetoin content of reaction mixtures was determined as acetoin by the method of Hill et al. (7). This method also measures 2,3-butanediol, but gas-chromatographic analysis of reaction mixtures and cultures by a slight modification (5) of the technique of Rogosa and Love (14) revealed the absence of 2,3-butanediol in all cases.

**Diacetyl and acetoin production by whole cells.** Cells were recovered from Elliker broth by centrifugation at 15,000 × g for 30 min at 2 C. The cells were washed once with 0.1 M potassium phosphate buffer (pH 7.0) and resuspended in phosphate buffer. The ability of isolated cells to produce diacetyl and acetoin was determined in the pyruvate assay mixture described by Speckman and Collins (17). This mixture contained 100 μmoles of sodium pyruvate, 0.02 μmole of thiamine pyrophosphate, 4.5 μmoles of magnesium sulfate, 0.1 ml of cell suspension, and 2.0 ml of 0.1 M phosphate buffer (pH 4.5) in a final volume of 3.0 ml. These mixtures were incubated at 30 C. The effect of pH on the reaction was determined by using a series of 0.1 M

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phosphate buffers to yield final pH values ranging from 4.5 to 7.0.

**Preparation of cell-free extracts.** Buffer suspensions of washed cells were cooled in ice and sonically oscillated for 15 min with a Branson Sonicator (model B110) operated at maximum amplitude. Cell debris was removed by centrifugation at 15,000 × g for 20 min at 2 C, and the cell-free supernatant fluid was recovered. The ability of this supernatant fluid to produce diacetyl and acetoin was determined in the pyruvate assay mixture described previously.

**Chemicals.** Sodium pyruvate and thiamine pyrophosphate were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Glutathione, 2-mercaptoethanol, dithiothreitol (Cleland's reagent), and sodium acetate were from Sigma Chemical Co., St. Louis, Mo.

**RESULTS AND DISCUSSION**

*L. casei* 393, when cultured in milk medium, utilized acetaldehyde and produced small amounts of ethanol and diacetyl (10, 11). Addition of citrate to the medium decreased the levels of diacetyl which accumulated by increasing the diacetyl reductase activity of the cells (3, 11). We have found pyruvate to be a precursor for the formation of diacetyl and acetoin by *L. casei* and that the presence of pyruvate in the culture medium enhanced diacetyl reductase activity, thus increasing the rate of diacetyl utilization by the test organism (3, 5).

Pyruvate, in levels of 16 μmoles per ml of medium, increased dry weight of *L. casei* incubated without shaking (Fig. 1). Stimulation was first evident after 18 hr, and the higher cellular dry weight of treated cultures persisted through 48 hr of incubation. Agitation of cultures at either 90 or 260 strokes/min caused a significant decrease in accumulated dry weight of *L. casei*. Addition of pyruvate overcame this effect (Fig. 1). At 90 strokes/min, cultures containing pyruvate displayed the same cellular dry weight as unagitated controls. At 260 strokes/min, pyruvate restored the accumulated cell weight to nearly the same level as that of the unagitated controls. We had previously demonstrated that inclusion of citrate into the medium significantly stimulated the accumulated dry weight of *L. casei* under agitated and unagitated conditions (4). This was confirmed in the present study and it was observed that, in the same molar concentrations, pyruvate was more effective than citrate in stimulating accumulation of *L. casei* cells.

The inhibition induced by agitation appeared to be related directly to increased contact with oxygen. Agitated cultures held in flasks tightly stoppered with rubber-lined screw caps showed much higher accumulations of cellular dry weights than agitated cultures contained in flasks closed with cotton plugs. In both cases, however, accumulation was still retarded relative to unagi-

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**FIG. 1.** Effect of agitation on accumulation of Lactobacillus casei. Pyruvate (PYR) was present in a level of 16 μmoles per ml of medium. Abbreviations: CONT, control cultures without added pyruvate; CPM, cycles per minute.

**FIG. 2.** Effect of pyruvate, citrate, and acetaldehyde on diacetyl and acetoin production by Lactobacillus casei. Each additive was present in a concentration of 16 μmoles per ml of broth medium. Incubation was for 24 hr at 30 C. Results are reported as diacetyl.
tated controls. Since it has been suggested that reduced growth rates in agitated cultures may be due to oxidation of enzyme sulfhydryl groups (5), the sulfhydryl-protecting reagents glutathione, 2-mercaptoethanol, and dithiothreitol were tested for their effect on accumulation of cells in agitated cultures. None of these reagents, when added in levels ranging from 2 to 20 μmoles per ml of medium, had any demonstrable effect on L. casei. Thus, factors other than oxidation of enzyme sulfhydryl groups appear to be responsible for retardation of Lactobacillus growth under aerobic conditions. Gilliland and Speck (6) observed no growth enhancement when catalase was added to cultures of lactic streptococci and lactobacilli.

Citrate, pyruvate, and acetaldehyde were tested for their effect on diacetyl and acetoin production by the test organism (Fig. 2). Agitation resulted in increased diacetyl accumulation with all compounds tested when compared to unagitated controls. Agitation also increased diacetyl accumulation by the test organism in the absence of additives. By far the greatest increase in diacetyl and acetoin accumulation occurred in agitated cultures containing pyruvate. Pyruvate also stimulated diacetyl and acetoin accumulation under still conditions, but the increase was much greater with agitation and the levels of diacetyl accounted for a much larger percentage of the total diacetyl plus acetoin levels under these conditions. Addition of combinations of pyruvate and citrate decreased the production of diacetyl and acetoin relative to the amount produced when pyruvate alone was present. Acetaldehyde had little effect when added either alone or together with pyruvate.

Based on a constant dry weight of cells, there was a much greater accumulation of diacetyl plus acetoin in agitated cultures compared to still controls (Fig. 3). Addition of either pyruvate or citrate to the broth medium resulted in both increased rate of synthesis and accumulation of total diacetyl and acetoin; pyruvate had a greater effect in this regard. With combinations of pyruvate and citrate, production of diacetyl plus acetoin was retarded relative to production in broths containing pyruvate alone. In nearly all cases, maximal levels of diacetyl plus acetoin were attained after 18 hr, but maximal cell populations were not reached until 24 to 36 hr. These observations indicate that pyruvate enhances both growth and diacetyl and acetoin formation by L. casei.

![Graph](image1.png)

**Fig. 3.** Effect of pyruvate and citrate on accumulation of diacetyl plus acetoin in broth cultures of Lactobacillus casei. A 16-μmole amount of each compound was added per ml of medium. Results were calculated as acetoin.

![Graph](image2.png)

**Fig. 4.** Effect of culturing time on diacetyl and acetoin formation by resting cells of Lactobacillus casei. Pyruvate and citrate refer to resting cells from cultures containing 16 μmoles per ml of the respective acid. Diacetyl plus acetoin was determined after incubation of isolated cells for 2 hr at 30 C. Results were calculated as acetoin.
Diacetyl and acetoin contents of cultures increased throughout the first 16 hr of incubation, indicating that these compounds arise as a result of the metabolism of cells during logarithmic and early stationary phases. Further, these results strongly imply that \textit{L. casei} converts pyruvate to diacetyl and acetoin. We have observed previously that cell-free extracts of \textit{L. casei} convert pyruvate to \(\alpha\)-acetolactate, which is decarboxylated subsequently to diacetyl and acetoin (5).

Isolated washed \textit{L. casei} cells suspended in buffer actively converted pyruvate to diacetyl and acetoin (Fig. 4). Resting cells, isolated at different intervals during the growth curve, yielded diacetyl plus acetoin production patterns nearly identical to those observed with actively growing cells in broth medium, with maximal production of these compounds being attained with cells harvested during the late logarithmic or early stationary phases (cf. Fig. 3 and 4). Cells harvested from broths containing pyruvate or citrate produced more diacetyl plus acetoin than did cells from control broths at all stages examined. In contrast to the situation with actively growing cultures, where agitation enhanced diacetyl production, cells isolated from agitated cultures produced less total diacetyl plus acetoin than did cells isolated from cultures incubated without agitation (Fig. 4). This indicates that the enhanced diacetyl and acetoin formation in agitated cultures resulted from something other than increased enzymatic ability to produce these compounds. Increased production of diacetyl and acetoin appears to be due to increased conversion of a diacetyl and acetoin precursor. One known precursor of both diacetyl and acetoin, \(\alpha\)-acetolactate, readily undergoes nonenzymatic decarboxylation to yield both acetoin and diacetyl (5,

![Graph](image1)  
**Fig. 5.** Effect of pH on diacetyl plus acetoin formation by isolated cells and cell-free extracts. Levels of diacetyl and acetoin, determined after incubation for 2 hr in the standard assay mixture, are expressed as acetoin. Pyruvate and citrate refer to cells harvested from media containing 16 \(\mu\)moles per ml of the respective acid.

![Graph](image2)  
**Fig. 6.** Production of diacetyl and acetoin by isolated \textit{Lactobacillus casei} cells in the presence of various levels of citrate. Acetoin plus diacetyl was determined after incubation for 2 hr at 30 C.

![Graph](image3)  
**Fig. 7.** Production of diacetyl and acetoin by \textit{Lactobacillus casei} cells isolated from media containing 16 \(\mu\)moles of pyruvate per ml as affected by the presence of citrate or acetate in the reaction mixture. Acetoin plus diacetyl was determined after incubation for 2 hr at 30 C.
8, 9, 16, 17), and increased oxygen content greatly stimulates this conversion (8). An alternative explanation is that aerobic conditions induce the cells to direct more pyruvate to oxidative breakdown reactions, such as diacetyl and acetoin formation, without increasing the enzymatic ability of cells to produce diacetyl and acetoin.

Although citrate did not enhance significantly diacetyl formation when added to broth cultures, resting cells isolated from citrate-containing broth showed an increased ability to produce diacetyl and acetoin from pyruvate. The optimum pH for diacetyl and acetoin formation from pyruvate was in the range of 4.5 to 5.5 for control, citrate-, and pyruvate-cultured resting cells (Fig. 5). However, the pH optimum for citrate-cultured cells was in the range of 4.5 to 5.0, whereas the range for control and pyruvate-cultured cells was between 5.0 and 5.5. In contrast, the optimum pH for diacetyl and acetoin formation in cell-free extracts from all cell types was 5.5 (Fig. 5). This indicated that the optimum pH for diacetyl and acetoin formation by whole cells reflected something other than the influence of pH on the enzymatic reaction. At the lower pH values, the charge on both the cells and on pyruvate would be neutralized, which may have facilitated the accumulation of pyruvate. Citrate can reduce the ionic charge of cells (12), and it was assumed to enhance the formation of diacetyl and acetoin by isolated whole cells by reducing cellular charge and thus facilitating pyruvate uptake. Addition of citrate to pyruvate-containing buffer suspensions of whole cells indicated that this was the case (Fig. 6). Although citrate itself was not converted to diacetyl and acetoin in this system, it stimulated diacetyl and acetoin formation from pyruvate. In concentrations ranging from 4 to 16 mM, citrate stimulated diacetyl formation by cells isolated from control, citrate, and pyruvate broths. In contrast, the presence of citrate inhibited diacetyl formation by cell-free extracts, indicating that it stimulated uptake of a precursor and not an enzymatic reaction (Fig. 7). Acetate, another compound which in itself was not converted to diacetyl or acetoin, stimulated formation of these compounds by whole cells and inhibited their formation in cell-free extracts (Fig. 7). This again indicated stimulation of uptake of a precursor.

Results presented herein revealed that pyruvate, as well as citrate, enhanced production of diacetyl in L. casei cultures, with pyruvate being much more effective in this regard. Aeration of cultures by agitation markedly enhanced diacetyl accumulation. Addition or omission of pyruvate or citrate and the control of culturing conditions would appear to be powerful factors to consider for the control of diacetyl formation in fermentations involving L. casei. Furthermore, these results point to the fallibility of extending results obtained with cell-free systems to the situation which exists in organisms in cultures. From a practical standpoint, our results point to the importance of considering the ionic charge on cells when attempting to alter the metabolic activity of microorganisms, particularly with regard to diacetyl and acetoin formation.

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