High-Efficiency Conversion of Pyruvate to Acetoin by Lactobacillus plantarum during pH-Controlled and Fed-Batch Fermentations†

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The influence of pH on the type and concentration of metabolites produced from pyruvate by Lactobacillus plantarum ATCC 8014 was examined in pH-controlled fermentors at pH values of 4.5 to 6.5. Specific growth rates, cell dry weights, and diacetyl concentrations were highest at pH 5.5, with values of 0.78 h⁻¹, 190 mg/liter, and 1.2 mM, respectively. While the conversion efficiency (millimoles of acetoin formed per millimoles of pyruvate utilized) was highest (94.6%) at pH 4.5, acetoin levels were similar (20 mM) between pH 4.5 and 5.5. Feeding stationary-phase cells exogenous pyruvate increased acetoin levels to 78 mM.

Lactobacillus plantarum is a homofermentative bacterium that ferments lactose and glucose almost entirely to lactic acid, but also produces trace (parts per million) quantities of acetoin, diacetyl, and 2,3-butanediol (13, 14). Relative insensitivity to bacteriophage attack; tolerance to low pH, heat, and salt; generally recognized as safe status in foods (3); and the ability to ferment more than 20 different sugars (4) recommend this species for use in biotechnological processes. Homofermentative species can be shifted to heterofermentative metabolism (16, 30). This suggests that the distribution of pyruvate to various end products, the key to the diversity of microbial end products (22), can be manipulated experimentally. Diacetyl and acetoin synthesis can be increased to improve the flavor of dairy products (5–8, 13–15), but true hyperproduction (i.e., millimolar rather than parts per million levels) would constitute a flavor defect and has not been examined. However, high-level production of acetoin would have applicability in other food systems. Microbiologically produced acetoin could be used directly, as a precursor to tetramethylpyrazine (10), or oxidized to diacetyl (21) for use as a natural flavor. It might also be reduced to 2,3-butanediol (21, 31) and used as a fuel. Thus, if pyruvate dissimilation could be shifted from lactate to the acetoin, diacetyl, 2,3-butanediol pathway, a number of industrially important fermentations might ensue.

The regulation of pathways from pyruvate to acetoin is complex and not entirely clear. The addition of citrate enhances diacetyl synthesis by inducing citrase and acetate synthetase (24) and increases acetoin synthesis in L. plantarum, but it is inhibitory to growth and cell mass synthesis (27). Pyruvate also stimulates diacetyl and acetoin synthesis (13, 15, 27). Lactobacillic starter cultures form acetoin and diacetyl in proportion to the concentration of pyruvate added (1). In L. plantarum ATCC 8014 test tube cultures, pyruvate utilization saturates at about 20 mM and results in acetoin production at 30% of the theoretical yield; the use of higher pyruvate concentrations simply increases residual pyruvate levels (27). The objectives of the studies reported herein were to determine the influence of pH on the distribution of pyruvate to lactate, diacetyl, and acetoin in L. plantarum and to demonstrate the temporal correlation of acetoin synthesis with pyruvate utilization. We also report the use of a regenerable fed-batch fermentation to increase final acetoin concentrations.

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MATERIALS AND METHODS

Culture conditions. L. plantarum ATCC 8014, obtained from the American Type Culture Collection (Rockville, Md.), was maintained in Lactobacillus MRS agar slants (Difco Laboratories, Detroit, Mich.) at 4°C and transferred monthly.

We used a modification of the medium of Craig and Snell (9), designated CS-T and containing (per liter of distilled water): yeast extract, 10 g; KH2PO4, 600 mg; KH2PO4, 600 mg; MnSO4·H2O, 20 mg; MgSO4·7H2O, 200 mg; FeSO4·7H2O, 10 mg; NaCl, 10 mg; Tween 80, 5 ml; and supplemented with 80 mM sodium pyruvate (Sigma Chemical Co., St. Louis, Mo.). The medium was adjusted to pH 4.5, 5.0, 5.5, 6.0, or 6.5 with HCl. The media were dispensed into screw-cap test tubes and flasks for inoculation and into the fermentor vessel. These were autoclaved for 20 min at 121°C.

The inocula for the fermentors were prepared by three successive transfers in CS-T medium with incubation at 37°C for 24 h. A 3 to 5% (vol/vol) portion of the third culture was used to inoculate a Mouse fermentor (Queue Systems, Parkersburg, W.Va.). The fermentor was operated at 200 rpm, 250-ml/min nitrogen overlay, 37°C, with pH maintained within 0.05 unit of the setpoint by acid (0.5 N HCl) and base (0.25 N NaOH) delivery pumps calibrated to monitor the total volumes used. Samples (20 ml) were drawn aseptically, and cell density was measured as A600 at 1-h intervals until the stationary phase, and at least every 6 h thereafter. Cells were then removed by centrifugation. During the pH-controlled fermentations, these supernatants were stored in tightly capped test tubes at −80°C until needed for gas chromatographic analysis.

During the fed-batch fermentation, maintained at pH 5.3, the supernatants were filtered (0.45-μm pore size) and analyzed immediately by high-pressure liquid chromatography as described below. When residual pyruvate levels plateaued, concentrated filter-sterilized sodium pyruvate was aseptically added to the fermentor vessel to bring the total...
pyruvate concentration to circa 90 mM. Concentrated sterile CS-T medium was fed at the point when no more acetoin was being produced and no more pyruvate was being utilized. Microscopic checks and periodic plating on MRS medium were used to verify culture purity. Specific growth rates were calculated from the linear portion of the growth curves. Cell dry weights were calculated from the $A_{600}$ by the method of Koch (23). Pyruvate utilization rates (millimoles per hour) and acetoin production rates (millimoles per hour) were calculated for periods of linear change. The acid addition rate (millimoles per hour) and pyruvate conversion efficiency (percent theoretical) were also calculated for each fermentation.

Metabolite analysis. Pyruvate and lactate were quantified with a Varian high-pressure liquid chromatograph (model 2210; Varian Corp., Walnut Creek, Calif.) equipped with a model 2010 solvent delivery system, a model 2050 UV detector set at 220 nm, a Polypore H cation-exchange column (22 cm by 4.6-mm inner diameter) equipped with a reverse-phase C$_{18}$ guard column (Brownlee Laboratories, Santa Clara, Calif.), and 0.069 N H$_2$SO$_4$ as the mobile phase (27). The detector was reset to 192 nm for acetoin determination (M. E. Meyer, M.S. thesis, The State University of New Jersey, New Brunswick, 1987). Diacetyl and acetoin levels in the pH-controlled fermentations were quantified by gas chromatographic analysis of culture supernatant extracts. The extracts were prepared by adding 1 μl of hexanal (as an internal standard) to 5 ml of culture supernatant, extracting with 2 ml of ethyl ether, blending on a vortex mixer for 1 min, and adding 1 g of anhydrous sodium sulfate to break the emulsion. The solvent layer was transferred to an air-tight tube and saved at 4°C until analysis. Extracts were injected onto a gas chromatograph (model 5830A; Hewlett-Packard Co., Avondale, Pa.) equipped with a model 18850A printing integrator and a flame ionization detector and modified to accept a wide-bore capillary column (30 m by 0.75-mm inner diameter) packed with Supelcowax 10 (Supelco, Inc., Bellefonte, Pa.). Satisfactory separation of diacetyl and acetoin was obtained when the oven temperature was held at 50°C for 7 min, increased to 120°C at 10°C/min, and then held at 120°C for 10 min. Diacetyl and acetoin concentrations were calculated from a separate correspondence factor versus concentration standard curves for each pH value examined as described elsewhere (A. H.-M. Hsu, M.S. thesis, Rutgers—The State University of New Jersey, New Brunswick, 1987).

RESULTS

pH-controlled fermentations. When L. plantarum was cultured in CS-T medium maintained at pH 4.5, 5.0, 5.5, 6.0, or 6.5 in separate fermentor runs, many differences in fermentation parameters were observed. Cell growth was rapid ($\mu = 0.78$ h$^{-1}$) at pH 5.5 and slowed markedly with decreasing pH (Fig. 1). The growth rate on the alkaline side of the optimum also declined, but not as severely. Cell dry weights of 24-h-old cultures followed a similar pattern. Diacetyl levels were also highest at pH 5.5, but only 1.2 mM was formed after 48 h of fermentation. No diacetyl was detected in cultures grown at pH 4.5, 6.0, or 6.5. The pH optimum for acetoin synthesis was much broader. While the pH 5.5 fermentation had the most acetoin at 24 h, by 48 h acetoin levels in fermentors at pH 4.5 and 5.5 increased to almost equal levels. Less acetoin was formed during fermentations maintained at pH 6.0 and 6.5. Even though there was considerable utilization of pyruvate not accounted for by increases in acetoin production at these pH values between 24 and 48 h, no acetate or ethanol was detected. Lactic acid production was similar (12.9 to 13.9 mM) in cultures maintained at pH 5.0 to 6.5, but no lactate was formed in the pH 4.5 culture. The relationship between residual pyruvate concentrations at 24 h and pH was inversely related to acetoin production. Pyruvate utilization and acetoin levels at 24 h were used to calculate conversion efficiencies (percent theoretical, based on 2 mol of pyruvate forming 1 mol of acetoin). The conversion efficiencies were 94.6, 46.0, 40.0, and 0% at pH 4.5, 5.0, 5.5, and 6.0, respectively. The conversion efficiency for the pH 6.5 fermentation could not be calculated because the residual pyruvate concentration was higher than the initial level.

Fed-batch experiments. Cells grew rapidly ($\mu = 0.48$ h$^{-1}$) and entered the stationary phase after 8 h during the first stage of a fed-batch fermentation maintained at pH 5.3. Pyruvate was rapidly utilized and acetoin was produced by stationary-phase cells, but pyruvate catabolism continued for almost 24 h after acetoin synthesis plateaued (Fig. 2). Lactate levels increased late in the exponential phase to 8 mM and remained constant until day 5. Low levels (0.8 mM) of diacetyl were formed during stage 1; no additional diacetyl synthesis occurred during the rest of the fermentation.

When pyruvate utilization plateaued, the culture was fed additional pyruvate (stage II). Acetoin synthesis resumed during this second period of pyruvate utilization, but leveled off at an acetoin concentration of 38 mM. Pyruvate catabolism continued without an increase in acetoin. No acetate or ethanol was detected at any point in this experiment. Lactate levels dropped at the end of stage II. Pyruvate added at 5.2 days was utilized fairly rapidly, but no marked increase in the rate of acetoin synthesis occurred during stage III. The cell density continued to decrease slowly owing to dilution by the acid used for pH maintenance.

While there was no change in acetoin or pyruvate concentrations between day 8 and 10, the requirement for continued acid addition for pH control indicated ongoing metabolic activity. Because the residual pyruvate concentration was high (57 mM), additional pyruvate was not fed. Instead, CS-T medium was prepared in concentrated form so that, upon addition to the fermentor, it would be diluted to single-stretch medium. Upon addition of the fresh CS-T medium, the culture grew exponentially ($\mu = 0.24$ h$^{-1}$) after a short lag period. Although cells grew more slowly than during stage I, they reached a higher final cell density. Lactate was produced late in the exponential phase. In the early stationary phase, pyruvate was catabolized and acetoin concentrations increased to a final concentration of 78 mM.

DISCUSSION

The catabolism of pyruvate by L. plantarum yielded primarily acetoin, low levels of lactate, and, under some conditions, diacetyl, but no acetate or ethanol under the conditions used in these studies. This contrasts with the report of Collins and Bruhn (7) that resting "Streptococcus diacetilactis" (now classified as Streptococcus lactis) cells convert 73% of pyruvate to acetate and only 10% to diacetyl and acetoin. Hickey et al. (21) reported that L. plantarum catabolizes pyruvate to acetate, acetoin, and lactate at a 6:2:1 ratio. Similar incremental acetoin-to-lactate ratios occurred during stages I and IV of the fed-batch experiment. The absence of acetate in our cultures is probably due to maintenance of dissolved oxygen at 0%, since the presence of oxygen is required for the production of acetate by L. plantarum (28).
The conversion efficiency (moles of acetoin produced per moles of pyruvate utilized) for acetoin synthesis was highest at pH 4.5. *L. plantarum* ATCC 8014 has both L(+) and D(−) NAD-dependent lactate dehydrogenases (nLDHs), EC 1.1.1.27 and EC 1.1.1.28, respectively (11, 17, 18). These nLDHs are nonallosteric (20) and have pH optima between 7.0 and 8.5 (11, 12, 25). Thus, at pH 4.5, intracellular acidification would decrease nLDH activity, no lactate would be produced (as was the case in the pH 4.5 fermentation), and pyruvate could be converted to acetoin with increased efficiency. The production of lactate in the fermentations at pH 5.0 to 6.5 reduced their conversion efficiencies.

During the fed-batch fermentation at pH 5.3, lactate accumulated only during periods of cell growth (stages I and IV) and was catabolized late in stage 2. Lactate catabolism suggests a scavenging metabolism in which lactate recycle to pyruvate provides an alternate energy source, as proposed by Snoswell (29). This conversion of lactate to pyruvate undoubtedly occurs via NAD-independent LDHs (iLDH; EC 1.1.99-) since nLDHs are not reversible under physiological conditions (17). Both L(+) and D(−) iLDHs have been reported for *L. plantarum* and have pH optima of 5.8 to 6.6 (12). Gasser’s (18) inability to detect iLDH activity in *L. plantarum* ATCC 8014 might have been caused by the use of an inappropriate electron acceptor in the in vitro assay.

High acetoin concentrations occurred at pH values of 4.5 to 5.5 after 48 h. The optima observed here were slightly broader than the optimal pH for acetoin and diacetyl production for *Lactobacillus casei*, which is between 4.5 and 5.0 (2). Indirect assay of acetoin production by dried cells of *Lactobacillus arabinosus* also demonstrated an acidic pH optimum (26). Acetoin production was much greater than diacetyl production, in agreement with previous reports (14, 21). Diacetyl reductase, which has optimal activity at pH 4.5 to 5.0 (2), may be responsible for the low diacetyl levels found at low pH values.

The apparent conversion efficiencies during periods of linear pyruvate utilization and acetoin production in the fed-batch fermentation were high (Table 1), but intervening periods when no acetoin was produced lowered the overall conversion efficiency for the 13-day fed-batch fermentation to about 28%. The rates of pyruvate utilization declined with
successive feedings of stationary-phase cells. Pyruvate utilization by *L. plantarum* is enhanced by an additional energy source (2, 14, 21) which may be depleted during the course of the fed-batch fermentation and regenerated during stage IV. Higher than theoretical yields can be attributed to endogenous pyruvate production. Lactate production in media lacking fermentable carbohydrates has been attributed to catabolism of yeast extract components (27). Since this must occur through pyruvate as an intermediate, the high residual pyruvate in the pH 6.5 fermentation and the higher than theoretical conversion efficiency in stage II of the fed-batch fermentation might be caused by a similar route.

Acetoin concentrations were increased by using fed-batch fermentation; to our knowledge, the 78 mM acetoin concentration observed here constitutes the highest level reported for *L. plantarum*. The conversion of acidic intracellular pyruvate to neutral acetoin may provide the cell with a detoxification mechanism as hypothesized by Harvey and Collins (19). The fed-batch fermentation suggests that this conversion is regulated. The mechanism of this regulation is the subject of ongoing investigations in this laboratory.

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LITERATURE CITED


