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Acetoin and Diacetyl Production by *Lactobacillus casei* subsp. *pseudoplantarum*

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ABSTRACT

The ability of *Lactobacillus casei* subsp. *pseudoplantarum* to produce acetoin and diacetyl (AD) was evaluated in succinate buffer (initial pH 4.4) containing sodium pyruvate, at 30°C. Cells grown in MRS broth containing pyruvate produced AD more rapidly than did an equal number of cells either grown in broth without pyruvate or even stored, after harvesting during logarithmic growth, in MRS broth in the presence of pyruvate for 120 minutes. One or more of the enzymes catalyzing formation of AD appears to be formed originally during growth in the presence of pyruvate. The rate of AD production by pyruvate-grown cells was exponential, being 0.08 μmoles mg dry wt^{-1} ml^{-1} min^{-1} during the first 30 min of the reaction. Storage of pyruvate grown cells at 7°C for 6 h in 0.3 mmol/L KH_2PO_4 buffer, pH 7.0 resulted in 77% loss of activity. Inclusion of 0.5 ml of MRS broth in the assay mixture led to a considerable increase in AD production by both cells grown in the absence and presence of pyruvate. Lactose slightly stimulated AD production in cells grown on lactose, whereas glucose had practically no effect on glucose-grown cells. Acetate and acetaldehyde reduced AD production. The effect varied with the compound used and strain studied. Of 7 concentrations of cetylpyridinium chloride tested for their effect on AD production, the least and most inhibitory concentration were 1 and 10 μg ml^{-1} of assay mixture.

Lactobacillus casei plays an important role in ripening and flavor development in many varieties of cheese. Several reports have been published on its ability to produce diacetyl and acetoin (*1,2,3,7*).

In most bacteria, diacetyl and its reduced forms - acetoin and 2,3-butanediol - are produced from sugar metabolism via pyruvate. However, in lactic acid bacteria, little of these, if any, are produced from carbohydrates unless an additional source of pyruvate is also present, e.g. citrate or pyruvate, which is not, however, used as an energy source. Acetoin is not produced from carbohydrate because all of the pyruvate produced by lactic acid bacteria during fermentation is reduced to lactate for regeneration of NAD from $NADH_2$ to continue the fermentation (*10*). If, however, pyruvate is exogenously supplied, its intracellular transport renders it so abundant that induced enzymes are immediately formed to eliminate its detrimental effect by converting it into neutral carbonyl compounds.

It appears that *L. casei* can not inducibly form pyruvic decarboxylase, the prerequisite for acetoin formation, during growth, with the pyruvate derived from glucose metabolism as the inducing substrate. *Escherichia coli,* in contrast, inducibly forms formic hydrogenlyase during growth, with the formate derived from glucose metabolism as the inducing substrate (*15*). However, *L. casei* can form acetoin and diacetyl in considerable amounts from exogenous pyruvate if a fermentable carbohydrate is present in the culture (*21*).

Gilliland et al. (*9*) observed that cells of *Leuconostoc citrovorum cremoris* grown in broth without citrate produced no diacetyl in milk acidified to pH 4.5 with citric acid. The inability of the cells to produce diacetyl is due to failure to form the inducible citrate permease (*10*) and citrate lyase (*13*), necessary for citrate metabolism, because of absence of citrate from the culturing medium.

Acetate alone was not converted to diacetyl or acetoin by *L. casei (3).* However, Branen and Keenan (*4*) reported that acetate enhanced diacetyl and acetoin formation by *L. casei* in buffer suspensions.

Milk may be contaminated with cationic detergents from dairy equipment inadequately rinsed after cleaning and disinfecting. Quaternary ammonium compounds up to 50 μg ml^{-1}, added simultaneously with pyruvate to a well-grown 48 h old culture of *Leuconostoc* in milk, did not inhibit acetoin production from pyruvate determined 2 h after the addition (*12*).

In this work, the ability of *L. casei* to produce acetoin and diacetyl as affected by culturing conditions, a source of energy and nitrogen and some metabolites was investigated. Experiments were also done to study the inhibition of acetoin and diacetyl production by a cationic detergent in the absence of interfering milk constituents.

MATERIALS AND METHODS

Organism

L. casei subsp. *pseudoplantarum* used in this study was isolated from salted raw milk and identified by Hegazi (*11*). The organism has been

maintained by subculturing every month at 30°C in a deep tube of MRS agar (*8*) and then storing it in the refrigerator.

Medium

The medium used was modified MRS broth and contained 1% glucose, but no triammonium citrate, sodium acetate and Tween 80.

Culture preparation

The organism was grown in the modified MRS broth with or without 0.5% sodium pyruvate for 17 h at 30°C.

Cell suspensions

One hundred ml cultures (0.1% inoculum, incubated for 17 h at 30°C) were harvested by centrifugation at 3000 × *g* for 20 min, washed once in 0.3 mmol/L KH_2PO_4 buffer, pH 7.0 and resuspended in the same buffer. The cells were added to the different reaction mixtures to give a dry weight of 0.4 to 0.5 mg ml^{-1} (*5*).

Storage in MRS broth in the presence of pyruvate

A technique similar to that described by Plummer (*17*) for induction of β-galactosidase of *E. coli* was adopted after slight modification. Cells harvested during exponential growth from 50 ml of MRS broth without pyruvate were resuspended in 5 ml of the same growth medium. Four test tubes containing 4 ml of the growth medium were set up. Five-tenths ml of the bacterial suspension was added; the test tubes were warmed to 30°C and at zero time 0.5 ml of sodium pyruvate solution (200 mmol/L growth medium) was added. Activity in one tube was stopped after incubation for 30, 60, 90 or 120 min by adding 0.5 ml of chloramphenicol solution (1 mg ml^{-1} water). A control was included by incubating the strain for 120 min in the absence of pyruvate. Each tube was centrifuged, the pellet was washed once in 0.3 mmol/L KH_2PO_4 buffer, pH 7.0, and resuspended in the same buffer. The cells were added to the reaction mixture to give a dry weight of 0.4 to 0.5 mg ml^{-1}.

Assays reaction mixture

The contained (in 3 ml): 200 μmoles of succinic acid (adjusted to pH 4.4 with 0.2 mol/L NaOH); 1.2 to 1.5 mg (dry weight) of whole cells and 50 μmoles of sodium pyruvate. According to the experiment, the mixture (3 ml) also contained 0.5 ml of MRS broth or 30 μmoles of glucose, 30 μmoles of lactose, 50 μmoles of sodium acetate, 11.4 μmoles of acetaldehyde (100 μg ml^{-1} reaction mixture) or 0.5 ml of cetylpyridinium chloride solution of known concentration. Reactions were initiated by addition of substrate and incubation occurred at 30°C. The reaction was stopped after the desired period by addition of 1 ml of 0.125 mol/L NaOH solution according to the technique of Speckman and Collins (*18*) and the content of diacetyl and acetoin was determined. Succinate buffer was chosen because succinic acid, as a metabolite, had no effect on acetoin formation by *Streptococcus cremoris* and *Leuconostoc* sp. in a mixed strain starter culture (*14*). Branen and Keenan (*4*) found the optimum pH for diacetyl and acetoin formation by whole cells of *L. casei* to be in the range of 4.5 to 5.5, which is close to pH 4.4 used in these experiments.

Determination of acetoin and diacetyl

As a rapid means of following the reaction, the total diacetyl and acetoin content of reaction mixture was determined by the colorimetric method of Westerfeld (*20*), as the sum of both, using a Bausch and Lomb Spectronic 21 Spectrophotometer at 520 nm. Results were calculated as acetoin.

RESULTS AND DISCUSSION

Culturing conditions

The ability of *L. casei* to produce acetoin and diacetyl (AD) in succinate buffer, pH 4.4, containing pyruvate is represented graphically in Fig. 1. Cells grown in broth containing sodium pyruvate produced diacetyl and acetoin more rapidly in the assay mixture than did an equal number of cells grown in broth without pyruvate (control), which is in agreement with the results of Branen and Keenan (*4*).

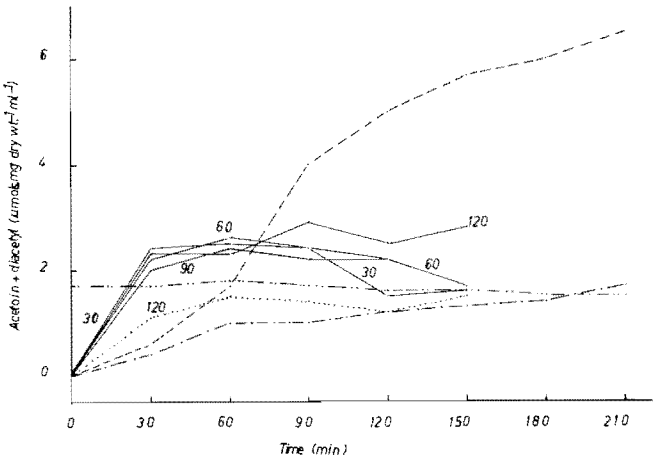

Figure 1. *Acetoin and diacetyl production from pyruvate by whole-cell suspensions of* L. casei *subsp.* pseudoplantarum. *Pyruvate grown cells ------; cells grown in the absence of pyruvate -.-.-.-.-; cells stored, after harvesting, in pyruvate containing MRS broth at 30°C for 30, 60, 90 and 120 min. ———; cells stored in MRS broth without pyruvate for 120 min. . . . . .; pyruvate-grown cells stored at 7°C for 6 h. -..-..-..-..-..*

The rate of AD production by pyruvate-grown cells was exponential, similar to that of allosteric enzymes, when the amount of AD was plotted against time. α-Acetolactate synthetase also appeared to be a homotrophic allosteric enzyme (*13*). In some experiments, AD were formed immediately after addition of pyruvate to the assay mixture, whereas in others it took a few minutes. The rate of AD production determined from the initial reaction velocity during the first 30 min from addition of pyruvate was 0.08 μmoles mg dry wt^{-1} ml^{-1} min^{-1} under the conditions of the experiments (average of 7 experiments). Since cells of *L. casei* grown in the absence of pyruvate produced less AD than those grown in its presence, it seemed possible that one or more of the enzymes catalysing formation of AD was present at a lower level of activity in cells grown in the absence of pyruvate. It also appears that the endogenous pool of nitrogen in these cells was not so abundant that enough amino acids were not available for formation of enzymes necessary for AD formation during the assay period. If, however, cells harvested during logarithmic growth were stored in MRS broth at 30°C for 30, 60, 90 and 120 min, in the presence of pyruvate, an increase in the ability to produce acetoin and diacetyl occurred. These cells showed approximately equal abilities to form AD, irrespective of storage period, with maximum production never reaching that of pyruvate-grown cells. There was generally no noticeable lag between addition of substrate and appearance of marked enzyme activity; products were readily formed, which indicates that the enzymes were present, but in a small amount.

Storage of pyruvate-grown cells at 7°C for 6 h in 0.3 mmol/L KH_2PO_4 buffer, pH 7.0 resulted in 77% of loss of activity, probably due to either inactivation or disintegration of preformed enzymes. Branen and Keenan (*3*) observed only 20% loss of activity in buffer at pH 7.0 at a temperature and for a storage period not stated. The en-

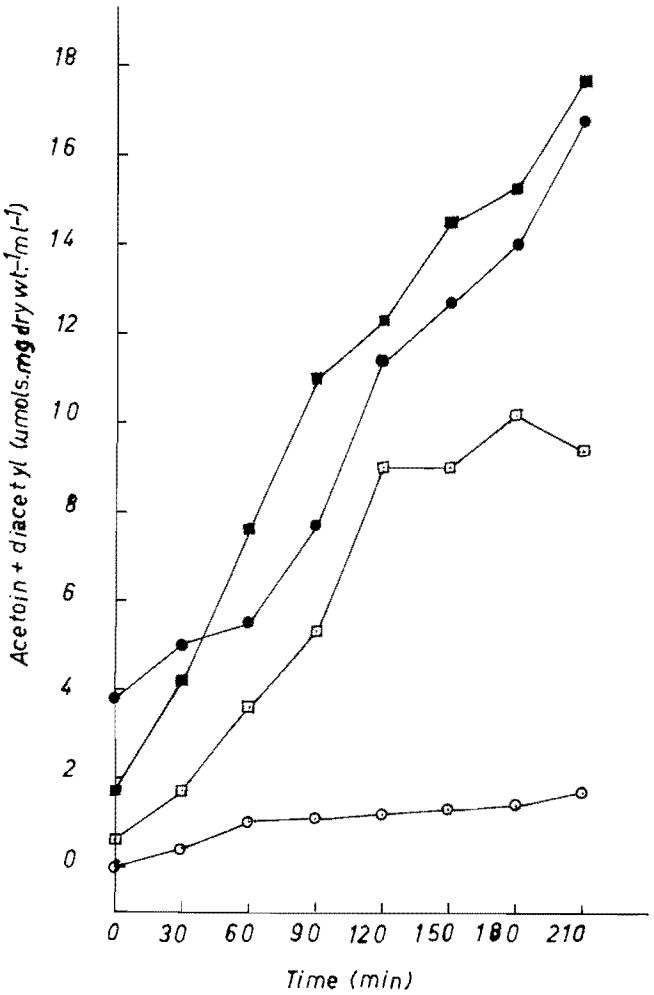

Figure 2. *Acetoin and diacetyl production from pyruvate by whole-cell suspensions of* L. casei *subsp.* pseudoplantarum. *Cells grown in the absence of pyruvate* ○——○; *cells grown in the absence of pyruvate + inclusion of MRS broth* ●——●; *pyruvate-grown cells* □——□; *pyruvate-grown cells + inclusion of MRS broth* ■——■.

zymes of pyruvate metabolism in *L. casei* are formed originally during growth of the microorganism provided pyruvate is present.

Effect of inclusion of MRS broth in the assay mixture on AD production

In an assay mixture without exogenous inclusions only small amounts of the enzymes were probably formed in the cells grown in the absence of pyruvate, since the strain studied depended only on its stored reserved for synthesis or induced enzymes. Inclusion of 0.5 ml of MRS broth in the assay mixture (Fig. 2) resulted in a considerable increase in the rate of AD production, probably due to formation of enzymes of pyruvate metabolism with concomitant production of AD at acid pH (initial pH 4.4). The importance of both a source of energy and nitrogen for enzyme formation has been clearly demonstrated in the induced formation of formic hydrogenlyase by resting cells of *E. coli (16)*. Even pyruvate-grown cells were also markedly stimulated by inclusion of MRS broth in the assay

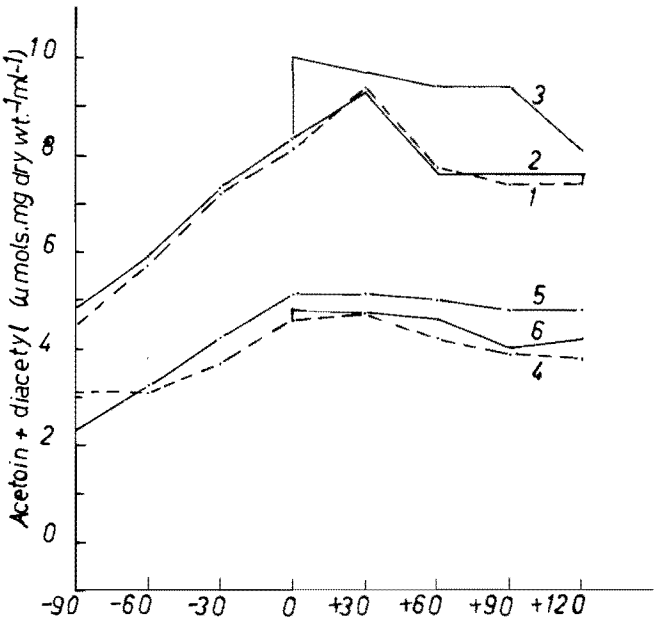

Figure 3. *Acetoin and diacetyl production by whole-cell suspensions of* L. casei *subsp.* pseudoplantarum *grown on either glucose or lactose in the presence of pyruvate, from: 1, pyruvate alone; 2, pyruvate + glucose; 3, acetoin + diacetyl after addition of glucose; 4, pyruvate alone; 5, pyruvate + lactose; 6, acetoin + diacetyl after addition of lactose.*

mixture as a result of a probable increase in the amount of enzymes of pyruvate metabolism. In agreement with these results, De Cardenas et al. (*7*) found that media were necessary to support the synthesis of enzymes, induced by the presence of pyruvate, in cells of *L. casei*. Branen and Keenan (*3*), on the other hand, reported that cells of *L. casei* harvested during logarithmic growth and cells grown in a medium containing pyruvate showed little difference in their ability to produce diacetyl and acetoin. The assay mixture they used in their experiments contained pyruvate, magensium sulfate and thiamine pyrophosphate.

Effect of carbohydrate on AD production

Inclusion of glucose in the reaction mixture (Fig. 3) did not accelerate the rate of AD formation in cells grown on glucose in the presence of pyruvate. Addition of glucose to cells actively metabolizing pyruvate caused an immediate increase in the level of AD followed by a decrease. Elevating the incubation temperature in the experiment from 30 to 41°C after 100 min from addition of pyruvate gave a similar result. Inclusion of lactose slightly stimulated AD production in cells grown on lactose in the presence of pyruvate, whereas its addition after 90 min had practically no effect. Cogan et al. (*5*), on the contrary, found total inhibition of acetoin production in glucose- and lactose-grown cells in *Leuconostoc lactis* NCWl by addition of glucose and lactose, respectively. Addition of glucose to cells actively metabolizing citrate gave similar results.

Effect of acetate and acetaldehyde on AD production

Acetate and acetaldehyde interfered with the use of pyruvate, resulting in partial inhibition of AD production

TABLE 1. *Acetoin and diacetyl production (μmoles mg dry wt^{-1} ml^{-1}) from pyruvate, in the presence of acetate and acetaldehyde, by whole-cell suspensions of* L. casei *subsp.* pseudoplantarum *333 C.*

Time in minutes	Pyruvate alone	Pyruvate + acetate	Pyruvate + acetaldehyde
0	0.3	0.8	0.6
30	1.3	1.3	1.2
60	1.7	1.5	1.6
90	4.4	2.3	2.9
120	5.4	2.2	3.8
150	6.3	2.3	4.4
180	6.7	1.9	4.5
210	7.0	2.2	5.7

TABLE 2. *Acetoin and diacetyl production (μmoles mg dry wt^{-1} ml^{-1}) from pyruvate, in the presence of acetate and acetaldehyde, by whole-cell suspensions of* L. casei *subsp.* pseudoplantarum *354 C.*

Time in minutes	Pyruvate alone	Pyruvate + acetate	Pyruvate + acetaldehyde
0	4.5	5.0	4.7
30	3.2	3.8	2.9
60	5.4	5.4	7.2
90	9.0	7.9	8.5
120	9.0	7.4	9.1
150	9.7	8.1	10.0
180	10.3	8.3	10.0
210	10.9	8.6	10.3

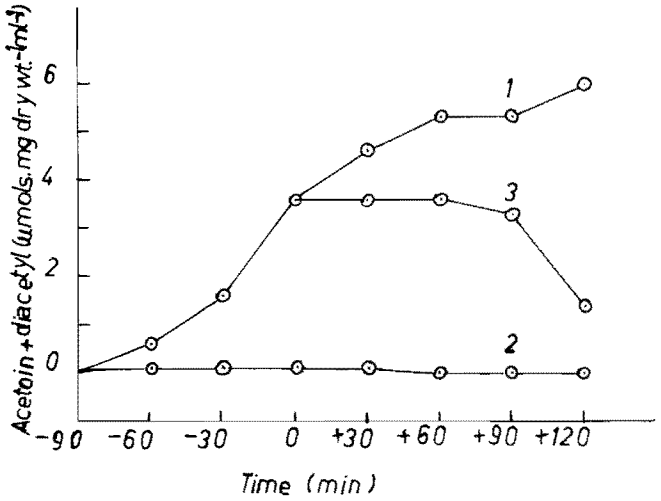

Figure 4. *Acetoin and diacetyl production by whole-cell suspensions of* L. casei *subsp.* pseudoplantarum *grown in glucose in the presence of pyruvate, from: 1, pyruvate alone; 2, pyruvate + cetylpyridinium chloride (50 μg ml^{-1}); 3, acetoin + diacetyl production after addition of cetylpyridinium chloride (50 μg ml^{-1}).*

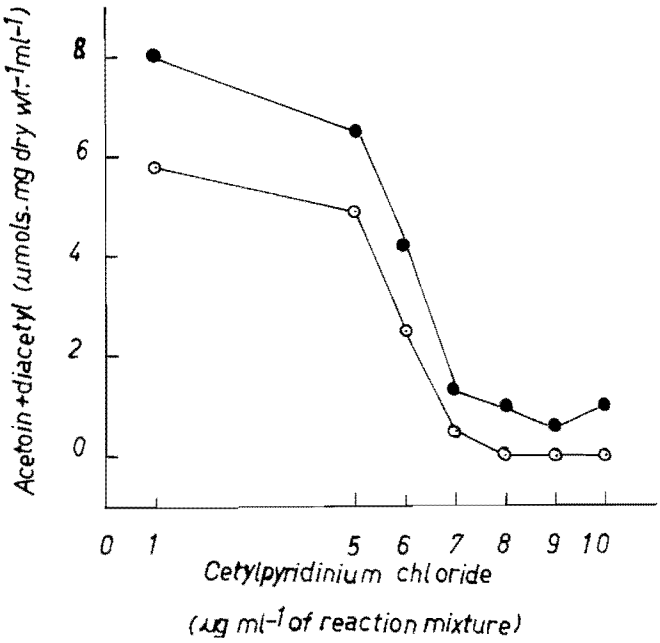

Figure 5. *Acetoin and diacetyl production from pyruvate, in the presence of various concentrations of cetylpyridinium chloride, by whole-cell suspensions of* L. casei *subsp.* pseudoplantarum, *grown on pyruvate, after: 210 min,* ●——●; *90 min,* ⊙——⊙.

after 90 min (Table 1). The AD level was reduced 71% by acetate after 180 min, and 34% by acetaldehyde after 90 min. The effect varied with the strain studied since in another strain of *L. casei* (Table 2), production was reduced only 21% by acetate after 210 min, whereas acetaldehyde had practically no effect. It seems that neither acetate nor acetaldehyde was involved in any condensation reaction leading to formation of diacetyl or acetoin. The findings of Collins and Speckman (*6*) that acetaldehyde (100 μg ml^{-1}) approximately doubled production of AD by growing a culture of *Leuconostoc citrovorum (cremoris),* probably by increasing the availability of hydroxyethyl-thiamine pyrophosphate and acetyl-coenzyme A, have not been realized in *L. casei* resting cells in these experiments.

Effect of cetylpyridinium chloride on AD production

Acetoin and diacetyl production was completely suppressed by the presence of cetylpyridinium chloride in the assay mixture (Fig. 4). This cationic wetting agent also had an inhibitory effect when added to cells actively metaboliz-

ing pyruvate. The level of AD decreased by increasing the concentration of cetylpyridinium chloride. The least and most inhibitory concentrations were 1 and 10 $\mu g\ ml^{-1}$ of reaction mixture, respectively (Fig. 5). These surface-active substances either dissolve in the constituents of the cell membrane or combine with them chemically, and thus change the surface of the cell to impair its essential functions (*19*).

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