Concentrated Cultures of *Leuconostoc citrovorum*¹

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Two single-strain cultures of *Leuconostoc citrovorum* were grown in a broth medium with automatic pH control. Culture concentrates were prepared by centrifugally harvesting the cells and resuspending them in 1/20th the original volume in 10% nonfat milk solids. The concentrates were stored in liquid nitrogen until analyzed. The maximum population attainable was approximately equal when cultures were grown at pH 6.0, 6.5, or 7.0 with sodium hydroxide or ammonium hydroxide as the neutralizer. Citrate was required in the growth medium for the cultures to be able to produce diacetyl subsequently in milk. At pH 6.0, the cells reached maximum population and ability to produce diacetyl. Organoleptic analysis by an experienced flavor panel showed a preference for cottage cheese creamed with a creaming mixture prepared with a culture concentrate rather than a normal culture. The culture concentrates maintained their viability and ability to produce diacetyl for at least 30 days when stored in liquid nitrogen.

Single-strain cultures of *Leuconostoc citrovorum* are employed for the enhancement and control of flavor in creamed cottage cheese (4, 6). *L. citrovorum* is grown in successively larger volumes of milk until a sufficient volume of culture is prepared to formulate the creaming mixture. The use of a concentrated suspension of *L. citrovorum* for the direct inoculation of the milk to be used in the creaming mixture would eliminate the in-plant build up of cultures and reduce the time required to produce desired flavor compounds. This would also minimize many problems involving culture activity and undesirable microbial contamination.

Concentrates of lactic streptococci for use in cheese manufacture have been successfully prepared and stored at subzero temperatures without loss in viability or biological activity (1, 7, 10).

The present investigation was undertaken to study the preparation of similar concentrates of *L. citrovorum*.

**MATERIALS AND METHODS**

**Cultures.** Two cultures of *L. citrovorum* (strains 3 and 28) were obtained from a commercial supply laboratory. The cultures were routinely propagated in sterile limus milk, by using a 1% inoculum and incubation at 25 C for 24 hr. The cultures were subcultured at least three time in the experimental medium prior to growing cells for preparing concentrated cultures. The cultures were stored in limus milk at 4 C.

**Culture growth with automatic pH control.** A 7.5-liter fermentor equipped with autoclavable electrodes in conjunction with an automatic pH controller (New Brunswick Scientific Co., New Brunswick, N.J.) was used to grow the cultures. Four liters of broth containing 2% trypone (Difco), 0.5% yeast extract (BBL), 1% glucose, and 0.5% sodium citrate was autoclaved at 121 C for 15 min and aseptically transferred into the sterile fermentor. The growth medium was inoculated with 40 ml of a fresh broth culture and incubated at 25 C. The pH was controlled at the desired level with either a 20% aqueous solution (weight per volume) of NaOH or NH₄OH. After the desired incubation time, the cells were concentrated and stored in liquid nitrogen by using the methods described by Peebles et al. (8).

**Cell population.** Colony counts were made with Elliker broth (Difco) plus 1.5% agar. Dilutions for plating were prepared by the technique of Peebles et al. (8), except that the initial dilution was blended for 2 min. Duplicate plates at each dilution were prepared and incubated at 32 C for 48 hr, after which all visible colonies were counted.

**Measurement of diacetyl production.** Frozen concentrates were thawed and diluted in 10% nonfat milk solids (NFMS) to a population equivalent to a culture of the same strain grown in 10% NFMS for 18 hr at 25 C. The pH was immediately adjusted to 4.4 to 4.5 with 15% citric acid. This required 4.2 ml of the citric acid per 100 ml of milk. The level of diacetyl in the acidified cultures was determined after incubation at 25 C by the method of Pack et al. (7). The amount of sample assayed depended on the level of diacetyl but was usually 10 or 20 g. The 10-g samples were purged with nitrogen for 1 hr and the 20-g samples were purged for 1.5 hr to ensure transfer of all the diacetyl to the hydroxylamine trap. A culture of the same strain

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grown for 18 hr in 10% NFMS was acidified and assayed in a similar manner for comparison.

**Organoleptic analysis.** Dry cottage cheese curd obtained from the North Carolina State University Creamery or a local dairy plant was creamed with a series of mixtures which were standardized to 16% fat with either 10% NFMS inoculated with a concentrated culture or a normal culture grown in 10% NFMS. The inoculated NFMS was incubated at 25 C for 6 hr at pH 4.4 to 4.5 prior to standardization and homogenization (2,400 psi). Salt (6.2%) was added to the homogenized creaming mixture before mixing with the dry curd. The creamed cottage cheese was stored at 4 C until analyzed. Members of the flavor panel were given a portion of each cheese and asked to assign a preference to each sample based on flavor and aroma.

**RESULTS**

**Citrate requirement.** Concentrated suspensions of *L. citrovorum* 3 prepared from cells grown at pH 6.0 in the broth medium without citrate did not produce diacetyl when inoculated into acidified milk (Table 1). There was no appreciable production of diacetyl until after the concentrate was subcultured three times in milk. The subcultures were prepared by using the equivalent of a 1% inoculum and by incubating for 18 hr at 25 C. The viable populations at the time of acidification show that the lack of diacetyl production by the concentrate was not due to insufficient inoculum. A culture concentrate prepared from cells grown in the same broth containing 0.5% sodium citrate produced a much greater amount of diacetyl in a 4-hr period than did the third subculture from the cells grown in the same broth without citrate (Table 1).

**Effect of pH on growth and diacetyl production.**

Cultures of *L. citrovorum* 3 grown in the sodium citrate broth at 25 C with 20% NaOH to control the pH at 6.0, 6.5, or 7.0 attained populations in 23 hr approximately twice those obtained without pH control (Table 2). Incubation of these cultures beyond the 23-hr period did not result in increased cell numbers. Preliminary studies have indicated that the cessation of growth was caused partly by depletion of nutrients. When the pH of the growth medium was maintained at 5.5, the population in 23 hr was about the same as that of the culture grown without pH control. The production of diacetyl by the *L. citrovorum* 3 concentrates decreased as the pH maintained during growth of the cells increased. Although the cells grown at pH 5.5 produced approximately 30% more diacetyl than an equal population of cells grown at pH 6.0, only one-half the population was attained by growing the cells at pH 5.5 as at pH 6.0. Therefore, more total diacetyl could be obtained from cells grown at pH 6.0 than from cells grown at pH 5.5.

Similar results were obtained when the pH of the growth medium was controlled with 20% ammonium hydroxide. Diacetyl production was also unaffected.

**Comparison with normal milk cultures.** The results in Table 1 suggested the possibility that cells of *L. citrovorum* grown in the sodium citrate broth would be more active than milk cultures with respect to diacetyl production. Results from experiments comparing diacetyl production of milk cultures to that of culture concentrates are presented in Table 3. For these studies, the culture concentrates were prepared from cells grown at pH 6.0 in sodium citrate medium with 20% NaOH as the neutralizer. For the analyses, the culture concentrates were inoculated into the acidified 10% NFMS to yield a population comparable to that of the normal milk culture. Cells from the *L. citrovorum* 3 culture concentrate produced considerably more diacetyl than did the cells in the normal milk culture of the same strain.

**TABLE 2. Growth of *Leuconostoc citrovorum* 3 at various pH levels and diacetyl production by subsequent concentrates**

| pH  | Time of growth | Plate count/ml | Diacetyl* (µg/g) | Plate count/g of acidified culture |
|-----|----------------|----------------|------------------|----------------------------------|
| 5.5 | 23             | 7.2 X 10^8     | 6.0              | 2.3 X 10^8                       |
| 6.0 | 23             | 1.4 X 10^8     | 4.4              | 2.1 X 10^8                       |
| 6.5 | 23             | 1.4 X 10^8     | 4.1              | 1.9 X 10^8                       |
| 7.0 | 23             | 1.2 X 10^8     | 3.7              | 2.2 X 10^8                       |

* Measured after 6 hr of incubation at pH 4.4 to 4.5.

*b Culture grown without pH control.

**TABLE 1. Diacetyl production in acidified milk by *Leuconostoc citrovorum* 3 cells after growth in broth with and without sodium citrate**

| Growth medium | Diacetyl* (µg/ml) | Plate count per ml at time of acidification |
|---------------|------------------|--------------------------------------------|
| Broth without citrate |                |                                            |
| Concentrate    | 0.0             | 5.7 X 10^8                                 |
| Subculture 1   | 0.2             | 3.2 X 10^8                                 |
| Subculture 2   | 0.1             | 2.8 X 10^8                                 |
| Subculture 3   | 1.4             | 2.4 X 10^8                                 |
| Sodium citrate broth |            |                                            |
| Concentrate    | 2.7             | 2.1 X 10^8                                 |

* Measured after 4 hr of incubation at pH 4.4 to 4.5.
The amount of diacetyl produced by the *L. citrovorum* 28 culture concentrate did not appear to be much greater than that formed by the normal milk culture of strain 28. However, by considering the difference in the number of cells in the samples for strain 28 at the time of acidification, the increased diacetyl production by the cells from the culture concentrate becomes more significant. These data also show *L. citrovorum* 28 to be considerably more active than strain 3 in production of diacetyl.

**Storage stability.** Culture concentrates of *L. citrovorum* were stable to storage in liquid nitrogen (−196°C). Table 4 shows results from analyses of an *L. citrovorum* 3 culture concentrate after various periods of frozen storage. There was no loss of viability or diacetyl production during the 30-day storage period.

**Flavor analysis of creamed cottage cheese.** Cottage cheese creaming mixtures were prepared using both normal milk cultures and culture concentrates of *L. citrovorum* 3, applied to dry cottage cheese curd, and subjected to analyses by an experienced taste panel. The results for two trials involving different lots of curd and creaming mixtures are shown in Table 5. In these experiments, the number of cells from the concentrated culture was adjusted to be equivalent to the population attained in the 18-hr milk culture. Cottage cheese made with the creaming mixture prepared with the concentrated culture showed superiority in diacetyl content, and the cheese was preferred by the taste panel. This preference was probably due to the higher level of diacetyl and, furthermore, indicated that no undesirable balance in flavor compounds resulted from the use of the concentrated culture.

**DISCUSSION**

When growing *L. citrovorum* cells for the preparation of culture concentrates, it is desirable to obtain the highest population possible. Although *Leuconostoc* species do not produce large amounts of acid, they do produce sufficient quantities to limit their growth in a broth medium. The results of this study have shown a twofold increase in the attainable population of *L. citrovorum* by controlling the pH at a level favorable for growth. The limitation on the population attainable may have been due to limiting nutrients or to the production of autoinhibitory metabolites other than acids.

The requirement for citrate in the growth medium for subsequent production of diacetyl by *L. citrovorum* concurs with results reported by Harvey and Collins (3), who found the citrate permease to be inducible. Harvey and Collins (2) also suggested that the citrate-splitting enzyme, citratase, is inducible in some organisms. The increased diacetyl production by culture concentrates prepared from cells grown at lower pH levels indicates a low pH optimum for synthesis of the enzyme system of *L. citrovorum* required for diacetyl production. Although the cells grown at pH 5.5 were more active in producing diacetyl than those grown at pH 6.0, the latter appeared to be more advantageous for cell population.

The direct inoculation of *L. citrovorum* culture concentrates into the acidified milk (pH 4.4 to 4.5) for preparing cottage cheese dressing does not permit growth of the culture; therefore, it is necessary to have a sufficient number of cells in the concentrate to produce the desired amount of flavor without further growth. The more active

| Table 3. Diacetyl production by normal milk cultures and concentrates of *Leuconostoc citrovorum* |
|---------------------------------------------------------------|
| Culture | Plate count/ml of acidified culture | Diacetyl (µg/g) |
|---------|-----------------------------------|----------------|
|         |                                   | 2 hr | 4 hr |
| *L. citrovorum* 3 (normal) . . . . . . | 2.9 × 10⁸ | 1.0 | 2.6 |
| *L. citrovorum* 3 (concentrate) . . . . . . | 2.9 × 10⁸ | 2.2 | 3.5 |
| *L. citrovorum* 28 (normal) . . . . . . | 4.3 × 10⁸ | 2.6 | 5.1 |
| *L. citrovorum* 28 (concentrate) . . . . . . | 3.3 × 10⁸ | 2.5 | 5.6 |

| Table 4. Storage stability of *Leuconostoc citrovorum* 3 concentrates |
|---------------------------------------------------------------------|
| Days stored (−196°C) | Diacetyl* (µg/g) | Plate count per g of acidified culture |
|---------------------|-----------------|---------------------------------------|
| 1                   | 7.0             | 3.1 × 10⁸                              |
| 10                  | 8.4             | 4.7 × 10⁸                              |
| 20                  | 8.0             | 3.8 × 10⁸                              |
| 30                  | 7.0             | 4.5 × 10⁸                              |

*Measured after 2 hr of incubation at pH 4.4 to 4.5

| Table 5. Flavor analyses of cottage cheese creamed with mixtures prepared with *Leuconostoc citrovorum* 3 concentrates |
|--------------------------------------------------------------------------------------------------------------------------|
| Trial | Culture | No. showing preference | Diacetyl (µg/g) |
|------|---------|------------------------|----------------|
| I    | Concentrate Milk culture | 4                   | 2.3 |
|      |                     | 3                   | 1.6 |
| II   | Concentrate Milk culture | 7                   | 2.6 |
|      |                     | 0                   | 1.3 |
production of diacetyl by the concentrated cultures offers an added advantage of requiring fewer cells than those in a milk culture for producing a given level of diacetyl. There was a certain amount of variation in the production of diacetyl by the two strains of *L. citrovorum*. This indicates that some strains offer an advantage over others for the preparation of culture concentrates capable of rapid diacetyl production.

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