Effect of Temperature on Growth and Diacetyl Production by Aroma Bacteria in Single- and Mixed-Strain Lactic Cultures

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Abstract

By the use of a differential plating medium, the growth of aroma bacteria (Leuconostoc citrovorum and Streptococcus diacetilactis) in mixed-strain lactic starter cultures was followed. Populations were greater at 30 C than at the temperature (21 C) normally used in the dairy industry for incubation of cultures for manufacture of fermented dairy products. At 30 C, peak level of diacetyl produced in mixed-strain lactic starters was lower than at 21 C; however, at the higher temperature there was an earlier initiation of the synthesis and the attainment of the peak level. Prompt cooling of cultures to 2 C inhibited diacetyl reductase activity and, consequently, loss of desirable flavor and aroma was prevented. The cooling, however, had to be carried out when the diacetyl level was high and before complete destruction of the flavor compound. By a careful combination of time and temperature of incubation and prompt cooling, the diacetyl flavor in milk cultures and Cottage cheese dressed with cultured cream could be stabilized and improved.

The flavor components of major importance in fermented dairy products are lactic acid and diacetyl. The former imparts the pleasant acid taste, while the characteristic butter-like flavor is attributed to the latter compound. Diacetyl in dairy products is formed by the fermentation of milk citrate by aroma bacteria, the Leuconostoc species and Streptococcus diacetilactis (4, 8). Leuconostoc species used in starter cultures have weak lactose-fermentative abilities (4, 14) and, due to the low pH optimum of citrate permease (6), milk citrate fermentation by these organisms occurs only after the pH is lowered below 6.0 by acid-producing species (Streptococcus lactis and Streptococcus cremoris). S. diacetilactis, on the other hand, is able to produce diacetyl from milk citrate even in the absence of acid-producing species, because of its lactose-fermenting ability (8).

In view of the associative growth conditions occurring in mixed-strain lactic starters, factors affecting the individual strain populations in the mixed-strain cultures play an important part in day-to-day manufacture of uniformly, high grade dairy products with respect to body, texture, and flavor. Incubation and storage temperatures are factors that can be easily controlled in day-to-day operations and are known to have important influences on associative growth.

Hammer and Babel (5) suggested that to maintain a proper balance between the lactic acid-producing and aroma bacteria, cultures should be incubated at 21 to 22 C. They state: "Temperatures such as 70 to 72 F have advantages of considerable importance. With definitely lower temperatures growth of the organisms is delayed and it is difficult to obtain rapid ripening, whereas with significantly higher temperatures over-ripening often occurs and conditions are more favorable for development of heat-resistant organisms that survive pasteurization of the milk. At relatively high temperatures growth of S. citrovorum and S. para-streptococcus is influenced greatly and a lactic culture that has been through a very few transfers at 98.6 F may no longer contain these organisms." Due to the lack of proper medium for the direct enumeration of aroma bacteria in mixed-strain starter cultures, reports on the effect of temperature on the growth of aroma bacteria under associative growth conditions have been largely speculative. In 1961, however, Galesloot et al. (3) described such a medium and more recently a similar differentiating medium for aroma bacteria in mixed-strain starters was described by Nickels and Leesment (9). In the present investigation, this latter medium was utilized to study the effect of in-

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cubation temperature on growth and diacetyl production by aroma bacteria present in mixed-strain starters. The use of low temperature as a means of preventing enzymatic destruction of diacetyl also was investigated, since the report of Elliker et al. (2) and Prill and Hammer (13) indicated that time-cooling was effective in stabilizing culture flavor.

Materials and Methods

Cultures. The four mixed-strain starter cultures A, B, E, and G used in this investigation were obtained from commercial sources. Culture A contained strains of *S. cremoris* and *Leuconostoc citrovorum*, and Culture B was made up of *S. lactis*, *S. cremoris*, and *S. diacetylactis* strains. The remaining two cultures contained *L. citrovorum* for aroma production in addition to acid-producers. The cultures were obtained as lyophilized powders and were added as direct seed to 11% reconstituted Matrix Mother Culture Medium (Galloway-West, Fond du Lac, Wisconsin) for the various experiments. Direct seeding of cultures was adopted to prevent strain dominance (15). *L. citrovorum* L was isolated from a mixed-strain starter culture and *S. cremoris* F_{10} obtained from Professor A. Zeilinger of Vienna, Austria.

For Cottage cheese dressing, a single-strain culture of *S. diacetylactis* 18-16 was used. The dressing was made as described by Elliker et al. (2).

N-L medium for the enumeration of aroma bacteria. The plating medium was made by mixing the following three formulations after tempering them in a 45 °C water bath:

a) Culture filtrate. A single-strain milk culture of *S. cremoris* incubated for 48 hr at 22 °C was filtered through a Whatman No. 1 paper. Ten-milliliter volumes of the filtrate dispensed into screw-cap test tubes were sterilized at 121 °C for 15 minutes.

b) Sodium citrate agar. The medium consisted of 2.0% Tryptone, 0.5% yeast extract, 0.25% gelatin, 0.5% glucose, 0.5% lactose, 0.4% sodium chloride, 0.2% trisodium citrate · 2 H<sub>2</sub>O, 1.0% calcium lactate · 5 H<sub>2</sub>O, 1.5% Bacto agar, and 100 ml distilled water. The pH was adjusted to 6.7 and the agar medium sterilized by autoclaving at 121 °C for 15 min.

c) Calcium citrate solution. Ten grams of sieved calcium citrate was suspended in 100.0 ml 1.5% (w/v) solution of carboxymethylcellulose (Uddevoll, CMC Cekol, MV, Uddevoll, Sweden). The dissolution of carboxymethylcellulose in distilled water was aided by warm-

- The agar medium was melted and after tempering at 45 °C in a water bath, the culture filtrate and the calcium citrate solution were added and mixed together. The ratio by volume of agar medium to culture filtrate and calcium citrate solution in the final mixture was 85:10:5.

- After pour plates (7 to 9 ml per plate) of suitable dilutions were made, the plates were incubated at 25 °C. Colonies of aroma bacteria are distinguished from the acid-producing species on this medium by the presence of a clear zone around the former due to the utilization of citrate. *S. diacetylactis* strains appear as medium-sized colonies with a clear halo, usually within two days. *Leuconostoc* appear as small colonies with a clear halo only after three to five days. This distinction, however, is variable and for positive identification it is necessary to inoculate colonies into litmus milk which is reduced by *S. diacetylactis* but not by *Leuconostoc*.

Diacetyl determinations. The method of Pack et al. (12) was used for estimating diacetyl in milk cultures and in Cottage cheese. A 20.0-ml volume of homogenate, prepared by blending 150.0 g of cheese with an equal amount of distilled water, was used for diacetyl determinations on Cottage cheese samples.

- For the study of temperature effect on diacetyl production-destruction patterns, cultures were incubated at the desired temperatures and samples were collected at various time intervals. The diacetyl concentrations were plotted against time to obtain time-course patterns at 21 and 30 °C, respectively.

- To determine effect of cooling on diacetyl stabilization, the milk cultures or dressed Cottage cheese were transferred to coolers and samples were collected at time intervals for diacetyl quantitation. The diacetyl concentrations were plotted against time, and comparisons were made between cooled and uncooled samples.

Hydrogen Peroxide-Catalase treatment of cultures. Details regarding this treatment have been described by Pack et al. (11). After treatment and initial incubation of cultures at 21 °C, experimental flasks were transferred to a cooler held at 2 °C; control flasks were held at 21 °C. After holding for five days, the diacetyl concentrations were determined and compared.

Results

The characteristic colonial appearance of the citrate-fermenting aroma species present in mixed-strain starter Culture B plated on the
N-L medium is shown in Figure 1. The clear halos surrounding the \textit{S. diacetilactis} colonies are clearly visible and the non-haloed colonies of \textit{S. lactis} and \textit{S. cremoris} also may be seen, though not as readily. \textit{Leuconostoc} colonies that appeared when the three other mixed-strain cultures were plated were identical, though somewhat smaller, especially when they first appeared, and with smaller halos.

Plate counts on N-L medium showing the growth of \textit{L. citrovorum} in nonfat milk in the absence of \textit{S. cremoris} are shown in Table 1. The \textit{Leuconostoc}, it may be seen, apparently were suppressed by growth with \textit{S. cremoris} and similar data were obtained with three other \textit{Leuconostoc} and \textit{S. cremoris} pair combinations. In another experiment, where mixed-strain starter Cultures A and B were incubated at 21 and 30°C, data (Table 2) indicated that the aroma bacteria grew better at 30°C than at 21°C, though in the case of the \textit{Leuconostoc}-containing culture, incubation for 24 hours was necessary to show the difference. Furthermore, rates of CO$_2$ evolution measured manometrically in milk by several \textit{Leuconostoc} strains were greater at 30°C. In view of this, it seemed advisable to examine the diacetyl production-destruction patterns at these two temperatures and typical curves for Cultures A and B at 21 and 30°C are shown in Figure 2. In both the cultures, a greater diacetyl concentration was obtained when the cultures were incubated at the lower temperature. However, the initiation of diacetyl synthesis was delayed considerably at the lower temperature. This also supported the plate count data suggesting better growth of aroma bacteria at the higher temperature.

Figure 3 illustrates the diacetyl production-destruction patterns in Culture E when samples of the milk cultures collected at various time intervals during incubation at 21°C were cooled and held at 2°C. Maximum diacetyl concentra-

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\hline
\textbf{Table 1} & \textbf{Numbers of \textit{Leuconostoc} present at various times} & \textbf{in single-strain culture and in combination with} \\
& \textit{S. cremoris} & \textit{as determined by plating on} \\
& \textit{N-L medium}\textsuperscript{a} & \\
\hline
\textbf{Hours at} & \textbf{Single-strain} & \textbf{\textit{L. citrovorum} in} \\
\textbf{21°C} & \textbf{\textit{L. citrovorum} L$_2$} & \textbf{\textit{plus S. cremoris}} \\
& \textbf{(X 10$^6$)} & \textbf{P$_{di}$ (X 10$^6$)} \\
\hline
0 & 0.00198 & 0.6 \\
4 & 0.00650 & 1.4 \\
8 & 0.0770 & 4.6 \\
12 & 1.10 & 10.0 \\
16 & 8.80 & 41.0 \\
24 & 130.0 & 31.0 \\
28 & 200.0 & \\
30 & 200.0 & 70.0 \\
\hline
\textsuperscript{a}Data provided by Ruth Brown, Department of Microbiology, Oregon State University.
\textsuperscript{b}Culture diluted at 0 time to about 2,000 cells/ml.
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\textbf{Table 2} & \textbf{Plate counts of aroma bacteria in mixed-strain} & \\
& \textbf{starter Cultures A and B incubated at 21 and} & \\
& \textbf{30°C. Samples were collected at the indicated} & \\
& \textbf{time intervals and plated on -N-L medium} & \\
& \textbf{Count (X 10$^6$) per ml of culture} & \\
\hline
\textbf{Hours} & \textbf{\textit{Leuconostoc} in} & \textbf{\textit{S. diacetilactis} in} & \\
& \textbf{mixed Culture A} & \textbf{Culture B} & \\
\hline
& \textbf{21°C} & \textbf{30°C} & \textbf{21°C} & \textbf{30°C} \\
0 & 0.014 & 0.014 & 0.009 & 0.009 \\
12 & 0.014 & 0.014 & 1.0 & 5.0 \\
18 & 1.0 & 1.0 & 9.0 & 16.0 \\
24 & 8.0 & 42.0 & 10.0 & 14.0 \\
\hline
\end{tabular}
\end{table}
tion and stabilization were achieved when the sample was cooled to and held at 2°C after incubation for 12 to 14 hours at 21°C. The peak level of diacetyl in the milk culture was also observed between 12 to 14 hours during incubation at 21°C. There was a slight increase in the diacetyl level when a six-hour sample was cooled to 2°C and held at that temperature; the 24-hour sample, however, showed negligible increase when cooled to and held at 2°C. Results of similar experiments conducted with Cultures E and G over extended periods of storage up to seven days at 2°C are shown in Figure 4.

Increase and stabilization of diacetyl flavor in Cottage cheese creamed with dressing containing S. diacetilactis 18-16 as compared to a control sample dressed with noncultured cream when held at 0 and 5°C, are illustrated in Figure 5. It may be seen that a greater increase and stabilization could be obtained at 5 than at 0°C.

Table 3 summarizes results of an experiment performed to study the effect of the combined use of hydrogen peroxide-catalase treatment and cooling on the diacetyl concentration and stability in a mixed-strain starter culture. It may be seen that the combined treatment provided greater stability than the hydrogen peroxide-catalase treatment alone.

Discussion

By use of the N-L differential plating medium, data were obtained which indicated that the growth of aroma bacteria in mixed-strain lactic starter cultures prepared by the direct seed process occurs more rapidly at temperatures higher than 21°C, the temperature normally used for culture incubation in the dairy industry. Thus, the industry practice of incubating cultured buttermilk and sour cream at 21°C need not be based on the belief that this temperature is required for the best growth of the aroma bacteria. However, it also has been shown that at temperatures above 21°C, diacetyl is destroyed more rapidly and also reaches a lower level at the peak of production.

The diacetyl curves for Cultures A and B revealed an earlier initiation of diacetyl synthesis at 30°C than at 21°C. This probably was due to

**Fig. 2.** The effect of incubation temperature on diacetyl production and destruction by mixed-strain starter Culture A at 21°C (open circles) and 30°C (solid circles) and by mixed-strain starter Culture B at 21°C (open triangles) and 30°C (closed triangles).

**Fig. 3.** The influence of culture age on effectiveness of cooling in stabilizing diacetyl in mixed-strain starter Culture E. The solid line is the control held continually at 21°C. The broken lines represent diacetyl levels found in cultures cooled to 2°C after 6, 12, or 24 hours.

**Fig. 4.** Diacetyl levels in 100-ml milk cultures of Starters E and G held continuously at 21°C (solid circles and triangles respectively) and cooled to 2°C after 18 hours (open circles and triangles respectively).
AROMA IN LACTIC CULTURES

7

6

5

4

3

2

1

5°C

O°C

CONTROL(5°C)

0 5 10 15 20 25 30

DAYS

Fig. 5. The effect of cooling on diacetyl enhancement in Cottage cheese creamed with cultured dressing. The top (—O—) and middle (—•—) curves indicate diacetyl contents in cheese dressed with the cultured cream, and the bottom (—Δ—) curve is that for cheese dressed with noncultured cream.

the faster rate of growth and acid production at the higher temperature and a consequent earlier reduction of pH below 6.0 necessary for the rapid uptake of citrate. Greater flavor destruction at 30 C might be due to enhanced diacetyl reductase activity at this temperature as compared to 21 C. Since it is likely that diacetyl synthesis and destruction take place simultaneously (10), the stimulated reductase activity would prevent the rapid build-up of diacetyl, especially during the last stages of the synthesis phase, due to the depletion of diacetyl precursors. As a result, during the final stages of synthesis, an apparent steady state between synthesis and destruction could occur, which is probably manifested as a plateau in the diacetyl production-destruction curve. At 21 C, on the other hand, there is a rapid accumulation of diacetyl, indicating that due to its reduced activity, the reductase system is unable to keep pace with synthesis; however, when the diacetyl precursors are completely exhausted the destructive phase is dramatically expressed. Consequently, at 21 C there occurs a sharp peak between the synthesis and reductive phases in both Cultures A and B. Confirmatory evidence for these possibilities would consist of determining the temperature optima for diacetyl reductase systems in aroma bacteria. Work along these lines is in progress.

The feasibility of arresting the loss of diacetyl by the diacetyl reductase activity in aroma bacteria through the prompt cooling of cultures was demonstrated by the results shown in Figures 3 and 4. As may be seen in Figure 3, the immediate cooling of Culture E at the peak level of the diacetyl in the culture not only stabilized the flavor over a storage period of four days but also resulted in one and one-half-fold increase in the diacetyl concentration. The increase in the diacetyl level at low temperatures was regarded to be the result of an oxygen-dependent enhancement of diacetyl production, as recently demonstrated by Lundstedt and Fogg (7). Figure 3 also indicated that cooling of the culture at the early stages of initiation of diacetyl synthesis did not result in a very great increase in the diacetyl level. This might have been due to the retardation of acid production at the low temperature as a result of which the pH of the medium was not lowered sufficiently for optimal citrate permease activity. Hence, due to the lack of sufficient diacetyl precursors, there was very little chemical synthesis of the compound. At the end of the destructive phase, due to the absence of diacetyl precursors, the cooling effect failed to reverse the flavor loss in the culture. As seen in Figure 4, cooling of Cultures E and G halfway in the destructive phase resulted in the immediate stabilization of the flavor, in addition to an increase in the diacetyl concentration beyond the maximum levels attained in uncooled control cultures.

The initial increase and the stabilization at the peak level of diacetyl in Cottage cheese dressed with cream ripened with \textit{S. diacetilactis} 18-16, when held at 5 and 0 C, respectively, indicated that the increase in the diacetyl level at low temperatures was due to the chemical or enzymatic conversion of diacetyl precursors to diacetyl. The combined use of cultured cream dressing ripened with \textit{S. diacetilactis} 18-16 and low temperature not only enhanced and stabilized the desirable diacetyl flavor in Cottage cheese (Figure 5) but, as reported earlier (15)
also prevented the growth of spoilage bacteria and enterotoxigenic pathogens. Regarding the cooling effect, it is noteworthy that Prill and Hammer (13) found that butter cultures ripened 16 hours at 21°C, then fortified with citric acid (0.15%) and chilled by packing in ice produced more diacetyl than control cultures held continuously at 21°C.

The combined use of hydrogen peroxide-catalase treatment and cooling afforded even greater enhancement and stabilization of diacetyl as compared to hydrogen peroxide-catalase treatment alone. In view of this, the combined use of cooling and peroxide-catalase treatment could offer a suitable method of maintaining full flavor in cultured dairy products without upsetting the operational schedules in a dairy plant. These methods also could be used to advantage in preparing cultures to be used for manufacture of starter culture distillate, now widely used as a flavor additive in margarine and other foods.

References