Growth of a Mixed Species Lactic Starter in a Continuous “pH-Stat” Fermentor

H.-E. PETTERSSON

Chemical Center, Division of Technical Microbiology, University of Lund, S-230 53 Alnarps, Sweden

Received for publication 4 December 1974

Optimum growth conditions for mixed species starter FDs 0172 in skim milk, whey medium, and tryptone medium at 25°C in a “pH-stat” continuous fermentor were investigated. Specific growth rate and productivity were found to be influenced both by the medium and the pH value. Highest productivity was found in skim milk medium between pH 5.5 and 5.9. Lactic acid-producing activity of harvested cells decreased by continuous cultivation at pH values lower than 5.9 to 6.1, especially in tryptone and whey medium. Bacterial balance, CO₂ production, and aroma formation were comparable to the control in skim milk and whey medium at any pH, but differed significantly for cells grown in tryptone medium.

Biomass production of starter bacteria has mostly involved some kind of neutralization of produced lactic acid in a batchwise procedure (4, 15, 16, 25, 29), followed by a centrifugation.

An alternative to this would be to perform the cultivation step in a continuous way. Continuous cultivation or propagation of starters and starter bacteria used in the dairy industry was first reported by Whittier and Rogers (31) and was first investigated by Wilkowske and Fouts (32). They used the lactic acid production, i.e., lowering of pH, to control the flow rate of fresh medium to the fermentor. This control system of neutralizing with fresh medium to keep a constant, predetermined pH could be classified as a pH-stat continuous cultivation (17). This method, together with chemostats with or without pH control, has been used for continuous cultivation of lactic starters and starter bacteria (1, 5, 10, 12–14, 17–19, 27). Much of this work has, however, been done on single-strain starter bacteria (10, 13, 14, 17–19).

Mixed species starters composed of multiple strains of Streptococcus lactis, S. cremoris, S. diacetylactis, and Leuconostoc cremoris (citrovorum) (BD starter) are used in Sweden, as well as in many other countries, for the production of various fermented products. Continuous propagation of mixed species/strain starters has been undertaken (27, 32), but more detailed studies of the behavior of such starters during continuous cultivations are needed before this method of cultivation could be applied to a process for the production of concentrated mixed species starters. This study was undertaken to see how a mixed species starter (BD type) behaves in a continuous fermentor (“pH-stat”) and to optimize this fermentation towards maximum productivity, with unchanged strain balance and other important metabolic activities compared to the control starter.

MATERIALS AND METHODS

Starter culture. One mixed species starter culture, FDs 0172, was used throughout this investigation. To keep the properties constant the starter was, on reaching our laboratory, inoculated in reconstituted skim milk (9.1% solids) at 2% (vol/vol) in stainless-steel probes and immediately frozen and stored in liquid nitrogen to serve as a stock culture throughout this investigation. When used for experiments, a probe with the inoculated frozen starter was thawed at 25°C for 10 min and incubated for 16 h at 22°C. The starter was subcultured twice before use.

Continuous culture apparatus and methods. An apparatus constructed according to Fig. 1 (9, 13) was used. The fermentor vessel was a small-scale (1.5-liter) laboratory glass fermentor (AB Biotec, Hägersten, Sweden). The conical shape (Fig. 1) allowed the cultivation of small volumes. A 320-ml cultivating volume was finally chosen in this study, which was found to be suitable with respect to the substrate demand. Fresh medium flow to the fermentor was controlled by a combination pH-electrode (Radiometer A/S, Copenhagen, Denmark) connected to a pH meter (pH meter 28, Radiometer A/S, Copenhagen, Denmark) and a titrator (TT 1 C, Radiometer A/S, Copenhagen, Denmark), which gave on-off signals to a peristaltic pump (LKB-Beckman Instrument AB, Villingby, Sweden).

According to a previous study on batch cultivations of the same starter, done by the author, a cultivation temperature of 25°C was chosen in this study. The
culture vessel and accessory equipment were sterilized in an autoclave before use. The combined reference-glass electrode was sterilized by immersion in a 70% (vol/vol) ethyl alcohol solution overnight and was rinsed with sterile water before being placed in position in the vessel. Sterile medium in the fermentor was inoculated (3% [vol/vol]) with the subcultured starter at 25°C. When the pH had dropped to 5.5, the continuous cultivation was started. This pH was chosen because initial trials showed that problems with casein coagulation in milk medium occurred at lower pH values.

Steady state (constant flow rate) was allowed to be reached after changes in constant pH values had been made (6 to 7 h). When steady state was established, samples were withdrawn for analysis. All experiments were performed at least in triplicate.

Media. Three different media were used in this study, according to a previous study on batch cultivation of this starter made by the author. (i) Skim milk medium with 9.1% solids (Semper, Sweden) was sterilized at 110°C for 10 min. This medium had a final pH of 6.6. (ii) Whey medium was prepared as follows. A 70-g amount of whey powder (spray dried) and 50 g of skim milk powder in 1,000 ml of water were treated with 0.5 g of papain (12,000 U/g; Merck, Darmstadt) at 30°C for 20 min, 50°C for 20 min, 75°C for 15 min, and 95°C for 15 min. The enzyme-treated suspension was cooled to 75°C, centrifuged (30 min, 5,000 x g), and filtered through a membrane filter (Seitz K7). After the addition of 5 g of yeast extract and 0.14 g of MnSO₄, the medium was sterilized at 121°C for 15 min. The final pH was 6.2. (iii) A third medium prepared according to Bergère (2) with 40 g of tryptone (Difco), 14 g of yeast extract (Difco), 90 g of lactose, and 0.14 g of MnSO₄ in 1,000 ml of water was also used. The lactose was sterilized in one-half the water volume at 115°C for 10 min, and the other components were sterilized at 121°C for 15 min. The components were mixed afterwards, giving a final pH of 6.5.

Centrifugation. Samples of the cell suspension from the harvest vessel (at +5°C) were intermittently centrifuged in a cooled (+5°C) centrifuge (Sorvall SS-3) at 34,000 x g with equipment for continuous centrifugation. To improve the separation of cells from the milk culture 1% of sodium citrate was added before centrifugation.

Analytical methods. (i) Bacterial estimates. Both microscopic and plate count methods were used to estimate the total number of bacteria. Bacterial cells in the milk medium were stained and counted directly in the microscope as described by Skar (28). Direct microscopic count in the transparent media (whey and tryptone medium) was performed, after dilution, in a counting chamber (Petroff-Hausser) with a phase-contrast microscope. All microscopic counts were true total counts, i.e., the single cells in the streptococcal chains were counted.

Total viable counts were determined with a pour-plate technique on calcium-citrate agar according to Nickels and Leesment (22). On this medium the bacterial balance between strains of S. lactis/cremoris, S. diacetylatexis, and L. cremoris (citrovorum) was estimated (22). Colonies identified as S. diacetylatexis or L. cremoris (citrovorum) on this medium were verified by picking and inoculating the corresponding colonies in lithmus milk (22). All counts were made in triplicate.

(ii) Lactic acid-producing activity. The acid-producing ability of cells, continuously harvested from the various experiments, was determined at 30°C by inoculating samples into reconstituted skim milk. The pH drop versus time was measured in the magnetically stirred culture by means of a six-channel registering pH apparatus (Radiometer A/S, Copenhagen, Denmark). The amount of inoculum was 2% (vol/vol). Relative lactic acid-producing activity (compared to liquid Nb frozen starter) was calculated from the time to reach pH 5.5 on the pH curve and the initial number of bacteria in the vessel according to a procedure of Bergère (2), which was shown to be applicable to mixed species starters.

(iii) Aroma production. The amount of diacetyl formed by the activity of S. diacetylatexis and L. cremoris (citrovorum) in the mixed species starter and starter suspensions was determined according to the method of Owades and Jakovac (Proc. Amn. Meet. Am. Soc. Brew. Chem., 1963, p. 22–25), modified for milk cultures by Pack et al. (24). The sample to be tested was inoculated in reconstituted skim milk (1% [vol/vol]) and incubated 16 h at 22.5°C. A 20-g amount of a 16-h culture was used for diacetyl
determination. The results were expressed as micrograms of diacetyl per milliliter in the milk culture after 16 h.

(iv) CO₂ production. The CO₂-producing ability of starters cultivated continuously with the pH-stat method was determined by the Warburg technique (30). The concentrated bacterial suspension (approximately 10¹¹ cells/ml) was diluted in sterile quarter-strength Ringer solution to a concentration of 10⁴ to 5 x 10⁴ cells/ml. An 0.5-ml amount of this diluted bacterial suspension was placed in the side arm of a Warburg cup, which held 2.5 ml of reconstituted skim milk. After connection to a manometer and tempering to 30.0 C, the suspension in the side arm was mixed with the skim milk. Manometric readings were taken every 30 min up to 5 h. All determinations were made in duplicate. The CO₂ production was calculated as microliters of CO₂ (30) liberated per cell, counted at inoculation time, during 4 h of incubation at 30 C.

RESULTS AND DISCUSSION

The continuous culture apparatus used in this study in its final construction (Fig. 1) worked almost without problems throughout the time of experiments (120 h). Some problems occurred, however, with coagulation of casein in the overflow tube, as well as some wall growth when the skim milk medium was used. The coagulation of casein in the overflow tube was almost entirely eliminated by pumping sterile milk backwards into the fermentor for a few minutes after each 24-h period of continuous cultivation. Steady state was then allowed to be reached (8 to 10 h). Another way to solve the problem with casein coagulating in the overflow tube would be to use a construction with a magnetic scraper according to Keen (9), but this was not easily applicable to this fermentor construction.

Wall growth in the fermentor vessel could not be totally eliminated, and this could perhaps have been due to the high growth rate in skim milk medium at pH 6.3 (Table 1; Fig. 2). The problem with coagulated casein became insurmountable after 6 to 7 days of continuous cultivation in skim milk medium with the construction of apparatus used (Fig. 1). None of these problems occurred in the other two media tested.

Influence of constant pH and medium on growth rate and productivity. Results concerning growth rate and productivity are presented and illustrated in Tables 1 through 3 and Fig. 2 and 3. They were calculated from determined values on average flow rates and bacterial counts (microscopic) at steady-state operation at the preset pH values during a cultivation period of up to 120 h.

In a continuous working fermentor at steady state the dilution rate (D) is numerically equal to the specific growth rate (μ) for the chemostat (8, 21). This relationship also holds true for continuous bacteriostat operation (23, 26). For the on-off control system the relationship is exactly the same, when the system approximates continuous flow. This was the case in the present study when the speed of the peristaltic pump was set at a sufficiently low value.

From Tables 1 through 3 and Fig. 2 and 3 it can be seen that average flow rate, and thereby D and μ, increased with increasing pH value in the three media tested. In tryptone medium the growth rates were higher than in the other two media, but the total number of cells was lower. This was probably due to the better buffering capacity of milk and whey medium, which caused a higher lactic acid concentration in these media than in tryptone medium at the same pH value. Steady state in whey medium at pH 6.3 was not achieved, since the pH of the uninoculated medium was around 6.2.

The high value for D and μ at pH 6.3 in skim milk medium could have been due to some wall growth in the fermentor, which was a problem when using this medium. This has been suggested by Larsen and Dimmick (11) and Keen

| Table 1. Continuous fermentation of reconstituted skim milk (9.1% solids) at various pH values |
|--------------------------------------------------|
| Parameter                                      | pH values |
|                                                 | 5.5  | 5.7  | 5.9  | 6.1  | 6.3  |
| Average flow rate (ml/h)                       | 84   | 120  | 172  | 203  | 359  |
| Dilution rate and specific growth rate (per hour) | 0.26 | 0.38 | 0.54 | 0.63 | 1.12 |
| Bacterial number (10⁶ cells/ml)                 | 1,650| 1,600| 930  | 410  | 110  |
| Aroma bacteria (%)                              | 16   | 14   | 20   | 20   | 12   |
| Bacteria production rate (10⁶ cells/liter/h)    | 434  | 601  | 501  | 261  | 124  |
| Relative lactic acid production activity (%)    | 88   | 74   | 100  | 100  | 24   |
| Gas production rate (10⁻⁴ ul/cell)              | 58   | 64   | 57   | 48   |
| Diacetyl production (µg/ml)                     | 1.1  | 1.6  | 1.9  | 1.4  |
Diacetyl production Dilution Relativelactic Average Bacterial number Gas

- **Table 2. Continuous fermentation of whey medium at various pH values**

| Parameter                          | pH values |
|------------------------------------|-----------|
|                                    | 5.5  | 5.7  | 5.9  | 6.0  | 6.1  |
| Average flow rate (ml/h)           | 90   | 120  | 134  | 184  | 196  |
| Dilution rate and specific growth  | 0.28 | 0.38 | 0.42 | 0.58 | 0.61 |
| rate (per hour)                    |       |      |      |      |      |
| Bacterial number (10^9 cells/ml)   | 1,800 | 850  | 545  | 190  | 170  |
| Aroma bacteria (%)                 | 17   | 7    | 4    | 11   | 17   |
| Bacteria production rate (10^9 cells/liter/h) | 510 | 319  | 229  | 109  | 104  |
| Relative lactic acid production activity (%) | 15 | 41   | 43   | 80   | 100  |
| Gas production rate (10^-8 μl/cell) | 32   | 38   | 42   |      | 30   |
| Diacetyl production (μg/ml)        | 1.2  | 1.1  | 0.8  |      | 1.9  |

- **Table 3. Continuous fermentation of tryptone medium at various pH values**

| Parameter                          | pH values |
|------------------------------------|-----------|
|                                    | 5.5  | 5.7  | 5.9  | 6.0  | 6.3  |
| Average flow rate (ml/h)           | 154  | 178  | 195  | 227  | 300  |
| Dilution rate and specific growth  | 0.48 | 0.56 | 0.61 | 0.71 | 0.94 |
| rate (per hour)                    |       |      |      |      |      |
| Bacterial number (10^9 cells/ml)   | 560  | 770  | 573  | 170  | 110  |
| Aroma bacteria (%)                 | 46   | 59   | 39   | 17   | 6    |
| Bacteria production rate (10^9 cells/liter/h) | 270 | 429  | 350  | 121  | 103  |
| Relative lactic acid production activity (%) | 18 | 6    |      | 6    | 50   |
| Gas production rate (10^-8 μl/cell) | 236  | 93   | 93   | 8    | 8    |
| Diacetyl production (μg/ml)        | 0.8  | 0.6  | 0.9  | 1.2  |      |

(10) as a factor which could influence the kinetics of growth.

Yield of cells, i.e., productivities, calculated as number of cells produced in the continuous fermentor per liter of culture volume per hour is presented in Tables 1 through 3 and Fig. 2 and 3. The maximum productivity was greater in skim milk medium and whey medium than in tryptone medium, to some extent due to the differences in buffering capacity between the media. In skim milk medium the maximum productivity was 600 × 10^6 cells/liter per h at pH 5.7 and at a dilution rate of 0.38/h, in whey medium it was 510 × 10^6 cells/liter per h at pH 5.5 and D equalled 0.28/h, and in tryptone medium it was 430 × 10^6 cells/liter per h at pH 5.7 and D equalled 0.56/h. Linklater and Griffin (14) stated that productivity should be greatest just above the coagulation point (pH 5.4 in their study) in a pH-stat continuous fermentor. This was not the case in this study, which could perhaps be explained by the greater sensitivity of the dominant *S. cremoris* strains in this starter to a low pH and increased lactic acid concentrations compared to *S. lactis* C10 used in their study (14).

Productivity maxima at about the same pH values found in this study were also found by Keen (10) with a *S. lactis* and a *S. cremoris* strain.

**Influence of growth medium and pH on bacterial balance in the mixed species starter.** Some trials with continuous cultivation of mixed species starters have been performed (1, 12, 27, 32), but no general survey of what happens to the balance between different strains and species in the starter has been carried out, except for simpler mixtures of bacteria (12).

The measurements used in this study to determine the bacterial balance (22) could not give an exact picture of the balance between individual strains and species, but together with other determinations of important metabolic activities it was considered to give a fully acceptable picture of important changes in the bacterial balance during the continuous pH-stat cultivation.

In Tables 1 through 3 the bacterial balance determined after a 5-day run on calcium-citrate agar (22) is expressed as percentage of aroma bacteria. The bacterial balance in the liquid N2 frozen culture, serving as inoculum, contained 87% *S. cremoris/lactis* and 13% aroma bacteria,
of which 50% was identified as *L. cremoris* (*citrovorum*) and the other 50% as *S. diacetylactis*.

In skim milk medium (Table 1) the percentage of aroma bacteria did not change significantly from the control at the pH values tested. However, the amount of *L. cremoris* (*citrovorum*) tended to decrease during the last 2 days of cultivation. Whey medium caused a small decrease in the amount of aroma bacteria at pH 5.7 and 5.9. In tryptone medium great changes in bacterial balance occurred, especially at pH values lower than 6.0, where the percentage of aroma bacteria (*S. diacetylactis*) increased considerably.

**Effect on important metabolic activities.** To determine if changes in bacterial balance influenced important metabolic activities, such as lactic acid-producing activity, gas (CO₂) production, and aroma formation (diacetyl), and if important changes in bacterial balance occurred that could not be detected by the relatively approximate method used for the balance determinations (22), these activities were also measured. From Tables 1 to 3 it can be seen that both pH and medium greatly influenced the ability of continuously harvested starter suspensions to produce lactic acid when inoculated in skim milk. The activity was greatest at the highest pH values, which is in good accordance with the results of Berridge (5).
The activity of cells grown in skim milk was greater than that of cells grown in whey medium, which in turn exceeded the activity of cells grown in tryptone medium. The value for lactic acid-producing activity at pH 6.3 in skim milk was probably unreliable, because of wall growth in the fermentor (compare the value for specific growth rate).

Gas production measured as CO₂ liberated per cell in reconstituted skim milk was affected by the changes in the amount of aroma bacteria and showed extreme values for cells continuously grown in tryptone medium at pH values lower than 6.0 (Table 3). In the other two media, where the proportion of aroma bacteria was quite normal, gas production did not change significantly compared to control (40 × 10⁻⁴ ml of CO₂ per cell) at any cultivation pH (Tables 1 and 2).

Diacetyl production decreased markedly when the starter was grown in tryptone medium (pH < 6.0) (Table 3), but was quite normal (1.5 ± 0.5 µg/ml) in the other two media at any pH (Tables 1 and 2). This reduced diacetyl production of cells grown in tryptone medium could have been due to the lack of citrate in this medium, which was shown by Gilliland et al. (7) to impair subsequent diacetyl production in milk medium.

Keen (10) observed a pH region between 5.5 and 6.0 where the production of soluble nitrogen compounds (i.e., effect of proteases) diminished. This could be one explanation for the decrease in lactic acid-producing activity of cells grown at the lower pH values in this study. Another possible explanation would be the increased inhibitory effect of lactic acid at lower pH values, especially on S. cremoris strains (3). A third possibility would be that, when cultivation was carried out at the lower pH values, the residence time in the fermentor increased, and this could have led to a selection of slow lactic acid-producing variants. Changes in the other metabolic activities measured can to some extent be explained by the changes in bacterial balance and lactic acid-producing activity measured.

From the results presented above the conclusions to be drawn are that pH-stat continuous cultivation of the starter used in this study (FDs 0172) should be performed in whey or rather skim milk medium at pH values >5.9 if the properties of the initial starter serving as inoculum are to be unaltered. The productivity values (Tables 1 through 3; Fig. 2 and 3) at optimum conditions for the two media were 501 × 10⁶ cells/liter/h in skim milk (pH 5.9) and 194 × 10⁶ cells/liter/h in whey medium (pH 5.9). This productivity in skim milk is almost equal to the overall productivity in a batch fermentation at constant pH (3, 4, 29). This means that substrate utilization is lower in the pH-stat continuous culture and the amount of suspension to be centrifuged is greater, compared to a batch production of starter concentrates.

The productivity could be increased further by using the chemostat principle with pH control outlined by Lloyd and Pont (17, 18), but in this case the increased accumulation of inhibitors could have changed the bacterial balance unfavorably compared to the pH-stat. This, however, has to be further investigated. Although the productivity was not increased in the pH-stat continuous cultivation compared to batch processes, production of mixed species starter concentrates by continuous fermentations should still offer an interesting alternative to batch methods, especially if it could be run for longer periods of time by choosing the proper media and apparatus construction.

ACKNOWLEDGMENTS

I wish to thank G. Sjöström for his valuable advice and stimulating discussions during this work, and also H. Ogura for his skillful technical and analytical assistance. M. Dostalek is thanked for valuable criticism in preparing the manuscript.

This work was financially supported by the Swedish Dairies’ Association.

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