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BACTERIOPHAGE DEVELOPMENT IN AN IMMOBILIZED LACTIC ACID BACTERIA SYSTEM.[1]

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SUMMARY

Bacteriophages were added to milk fermented by Streptococcus raffinolactis cells immobilized in calcium alginate. Beads containing the immobilized streptococci were used for five consecutive fermentations; pH, free cell and bacteriophage counts were estimated. Free cells increased from 5 x 10^6 to 3 x 10^8 per mL of milk, over the successive fermentations. Addition of bacteriophages reduced the free cell count by almost 1000 after 3 fermentations, but a gradual increase occurred subsequently. Bacteriophages were inoculated at 100 per mL and gradually attained 5 x 10^9 per mL in the system. Rinsing of the system did not have a substantial influence on free cell or phage counts. Presence of bacteriophage reduced slightly the acidification rate in the system.

Bacteriophage numeration by two layer agar method gave better results than by most probable number (MPN). MPN counts were greatly influenced by S. raffinolactis inoculation level.

INTRODUCTION

Immobilized microorganisms have been proposed for many industrial applications (Linko and Linko, 1984) including the lactic acid bacteria (Linko, 1985; Champagne and Boyaval, 1986). Whey (Roy et al, 1987) and cream (Champagne and Côté, 1987) can be fermented by immobilized lactic cultures. Immobilized bacteria can also be used as a continuous source of inoculant for dairy fermentations. Thus Cavin et al (1985) report continuous production of propionibacteria for emmenthal flavors while Prévost et al (1985) as well as Prévost and Divies (1987) have demonstrated that yoghurt and cheese can be manufactured by using cells released in a bioreactor containing immobilized lactic acid bacteria.

Since cells are immobilized, they can be used for extended periods. Thus the continuous use of a given culture raises the possibility of bacteriophage contamination which can be quite rapid in large plants (Lawrence et al, 1978). Bacteriophage attack constitutes a major problem in the dairy industry (Lawrence et al, 1976; Huggins, 1984). Many precautions are taken to prevent bacteriophage attack (Shaw, 1983), one of them being culture rotations (Huggins, 1984; Klaenhammer, 1984).

Streptococci immobilized in calcium alginate are protected from bacteriophages (Steenson et al, 1987); however, free cells in the system are not. This population can be significant (Prévost et al, 1985; Prévost and Divies, 1987) and fermentation rates could be affected. Therefore, immobilized systems used for the purpose of continuous inoculation could be vulnerable to phage contamination. Since there has been no report of phage attack on propionibacteria (Gilliland, 1985) the process proposed by Cavin et al (1985) would appear unaffected. However, there are reports of bacteriophage infection in yoghurt strains (Accolas et al, 1980) and mesophilic lactic acid bacteria are very susceptible to bacteriophages (Babel, 1976; Cogan, 1980).

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[1] Contribution # 099

The aim of this work was thus to study bacteriophage development in an immobilized lactic acid bacteria system. We also report a comparative study of plaque and most probable number techniques for bacteriophage numeration.

MATERIALS AND METHODS

1. Media.

Low heat skim milk powder was rehydrated at 11% solids ($^{W}/w$) and heated at 115°C for 10 min (SM medium). For most probable number (MPN) numeration of bacteriophages, 0.01% brom cresol purple (BCP) was added to SM. M-17 broth or agar (Bio Carr) were sterilized at 115°C for 20 min. and 1 mL of sterile 1 M $CaCl_2$ (121°C, 15 min.) was added to 100 mL of M-17 media. Elliker agar (Difco) was supplemented with 0.1% K_2HPO_4 and sterilized at 121°C for 15 min. Sodium alginate (BDH) was rehydrated at 2% solids and sterilized at 121°C for 15 min. Dilutions were performed in sterile peptone (0.1%) water.

2. Biological.

Streptococcus raffinolactis[1] CRA-1 was maintained on SM medium. Two transfers per week were performed by inoculating at 1% ($^{V}/v$) and incubating at 21°C until a pH of 4.7 was reached (approximately 15 hours). Cultures were maintained at 4°C between transfers.

3. Cell immobilization.

One hundred mL of M-17 broth were inoculated with 0.1 mL of a fresh culture of S. raffinolactis CRA-1 and incubated at 22°C for 20 hours. The culture was then centrifuged at 2000 G for 10 min. and cells resuspended in 11 mL of sterile peptone (0.1%) water which gave a 1.1 x 10^{10}/mL cell density. Five mL of cell concentrate were mixed to an equal amount of sterile alginate and added dropwise to 0.1 M $CaCl_2$. The beads were allowed to harden for 1 h in the $CaCl_2$, which was then removed by decantation. They were then washed once with 10 mL of sterile peptone (0.1%) water. Cold SM (4°C) was added to the beads which were then kept at 4°C overnight.

4. Fermentations.

One hundred mL of SM (30°C) were added to the beads (obtained from 5mL of concentrated cell suspension) and incubated at 30°C for 2 h in a bottom baffled erlenmeyer (Bellco) under 100 RPM agitation. In some flasks 100 bacteriophage per mL were added at the beginning of the fermentation. Following the fermentation, the milk was then removed and 100 mL of fresh SM added. Prior to addition of fresh SM, some beads were rinsed by adding 100 mL of sterile peptone water, mixing for 10 seconds, and removing the peptone water prior to addition of fresh SM. Following this procedure, the immobilized bacteria were used for 5 successive 2 h fermentations. Samples were taken at the end of each fermentation for pH determination, bacteriophage and free bacteria counts.

5. Bacterial and phage counts.

Free S. raffinolactis cells were estimated by plating on Elliker agar supplemented with 0.1% K_2HPO_4. This medium did not permit multiplication of our phage thus eliminating the possible interference of bacteriophage present in some samples. Cell counts were as good as those obtained on M-17 (samples of S. raffinolactis gave 125 x 10^6 per mL on M-17, while Elliker agar gave 118 x 10^6 per mL).

A comparison of plaque and most probable number (MPN) methods of phage numeration was performed. Plaque counts were obtained by standard two layer agar (Potter and Nelson, 1952) in which 0.5 mL of sample and 0.05 mL of culture were added to 5 mL of M-17 agar (with $CaCl_2$),

[1] Under the new Bergey's classification (1986); formerly S. cremoris.

mixed, and poured over 15 mL of pre-solidified M-17 ($CaCl_2$). Lysis zones were counted followinga 24 h incubation at 30°C . In the most probable number (MPN) method, 1 mL of sample and 0.1 mL of culture were added to 10 mL of SM (with BCP); five tubes of each dilution were inoculated. Various inoculation levels were studied: 0.1 mL of fresh culture and serial dilutions $^1/10$, $^1/100$ and $^1/1000$ of the culture. This permitted initial S. raffinolactis populations of 5 x 10^3, 5 x 10^4, 5 x 10^5 and 5 x 10^6 per mL of SM (BCP). Following a 24 h incubation at 22°C, samples showing pH values 0.4 units superior to those of controls were considered as being positive for phage.

Results are the average of at least three separate trials.

RESULTS AND DISCUSSION

1. Bacteriophage numeration.

Numeration of bacteriophages can be performed by MPN or two-layer agar (TLA) techniques. Although TLA generally permits higher recovery rates (Turner and Nelson, 1951), MPN occasionally gives better results (Potter and Nelson, 1952). We therefore examined which method was the most appropriate for our specific host-phage system.

It was shown that inoculation level of agar influences results in TLA (Turner and Nelson, 1951). We found this to be also the case for MPN numeration of bacteriophages (Figure 1); inoculation of 5 x 10^4 S. raffinolactis per mL gave best MPN results. However TLA gave higher counts than all MPN. Thus our phage stock was estimated at 16 million per mL with TLA while the highest MPN titration gave 8 million per mL (Figure 1). We therefore opted for TLA numeration of our phage.

2. Free cells.

Bacterial cells are released from the surface of the alginate and can multiply in the medium (Prévost and Divies, 1987). When no bacteriophages were added, we found 5 x 10^6 free cells per mL following the first fermentation and repeated use of the immobilized cells over 5 successive fermentations permitted free cells to attain 4 x 10^8 per mL (Figure 2). Rinsing of the system does not lower very substantially the amount of free cells (Figure 2). Thus it appears that growth occurred on the surface of the beads during repeated use of the beads which permitted higher levels of cell release. Our results are in agreement with those of Prévost and Divies (1987) who found that 1.5 x 10^8 per mL free cells were obtained under steady state.

The presence of bacteriophage in milk had an important influence on levels of free cells inthe system (Figure 2). After 3 fermentations the free cell count was reduced to 6 x 10^5 per mL. Under classical fermentations, the amount of cells that survived a bacteriophage attack was only 4.3 x 10^2 per mL (Figure 3). Thus in an immobilized cells system the amount of free cells that follow a bacteriophage attack is at least 1000 times higher. This could be related to continuous release of cells from the beads. Cells at the surface of the beads were probably gradually attacked resulting in reduced levels of cell release for the three first fermentations. The gradual build up observed during subsequent fermentations, might be related to establishment of a phage resistant population.

3. Bacteriophage growth.

In a free cell system, bacteriophage count passed from 10^2 per mL to slightly over 10^7 per mL in two hours (Figure 3). The same growth rate was observed in an immobilized cells system. Thus in the first fermentation with immobilized cells, milk was also inoculated with 10^2 phages per mL which reached 10^7 per mL after two hours (Figure 4). Repeated use of the immobilized cells resulted in phage buildup in the system, up to 6 x 10^9 per mL. We determined that approximately 3 mL of medium remained in the flasks following decantation. Thus fresh sterile milk added to the system was contaminated at a 3% level. Rinsing of the system permitted

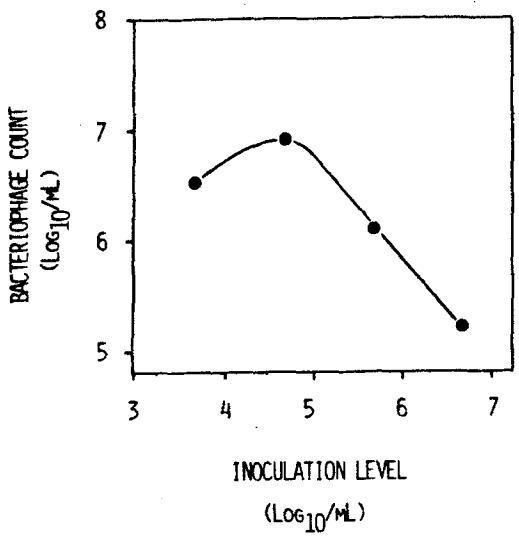


Figure 1: Effect of culture inoculation level on MPN bacteriophage count.

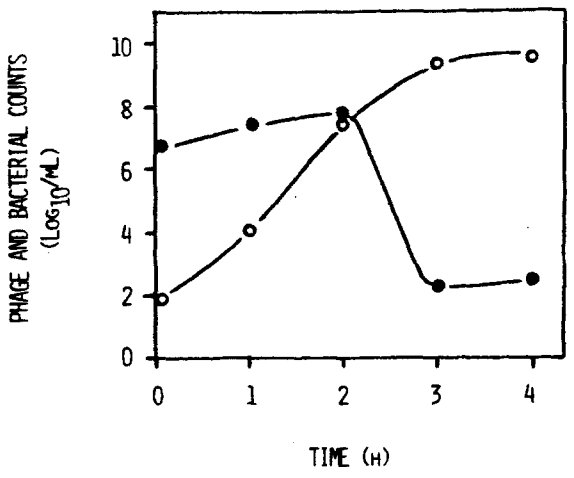
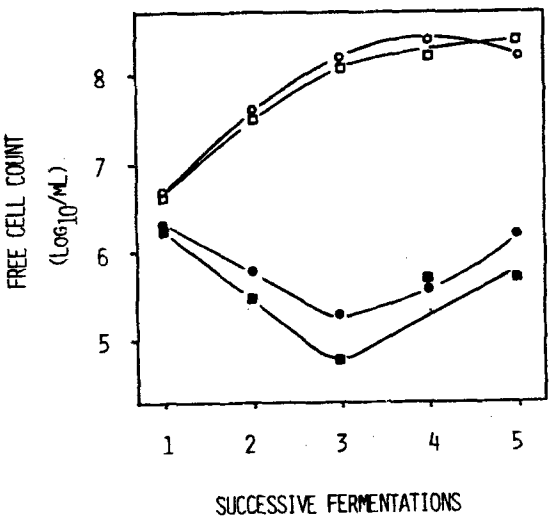


Figure 3: Multiplication of bacteriophage in a classical free cell system.
(O) Bacteriophage
(●) S. raffinolactis

Figure 2: Effect of bacteriophage and rinsing on free cell counts in an immobilized lactic acid bacteria system used for 5 consecutive fermentations.
(O) No bacteriophage, unrinsed system.
(●) Bacteriophage added, unrinsed system.
(□) No bacteriophage, rinsed system.
(■) Bacteriophage added, rinsed system.



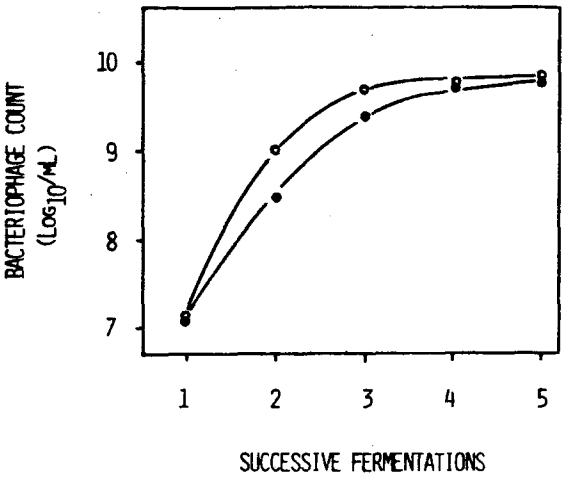


Figure 4: Effect of rinsing on bacteriophage counts in an immobilized lactic acid bacteria system used for 5 consecutive fermentations.
(O) unrinsed.
(●) rinsed.

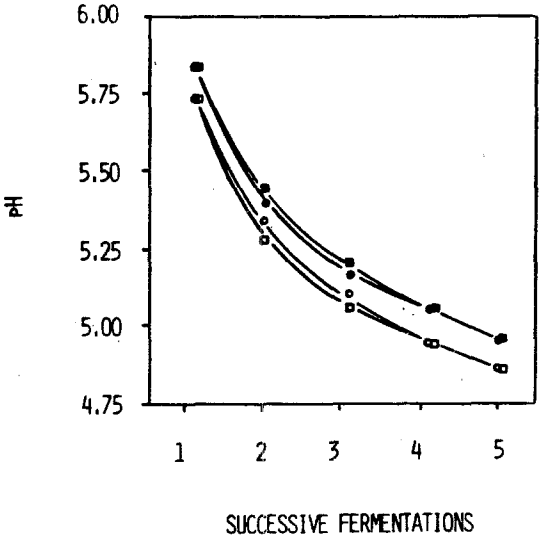


Figure 5: Effect of bacteriophage and rinsing on acidification over 5 consecutive fermentations.
(O) No phage, unrinsed.
(□) No phage, rinsed.
(●) Phage added, unrinsed.
(■) Phage added, rinsed.

to slightly reduce growth of phage in the three first fermentations, but did not show any effect subsequently to the third fermentation (Figure 4). Since phage multiplication in a single fermentation was 100,000 fold, the rinsing level we practiced was insufficient. Thus, it would have required at least 4 rinses after each fermentation to prevent phage buildup in the system.

4. Acidification rates.

Reutilization of immobilized cells resulted in an increase of the acidification rate of milk (Figure 5). Growth of bacteria occurs in alginate gels (Ohlson et al, 1979; Cavin et al, 1985) which could explain this increased activity. When bacteriophages were absent a gradual increase in free cells occurred during repeated use of beads (Figure 2) and could also contribute to the higher activity. Since rinsing of the system did not greatly influence free cell counts (Figure 2), acidification levels of rinsed and unrinsed systems were similar (Figure 5). Presence of bacteriophages did reduce acidification rates (Figure 4). Although bacteria inside the gel are protected from phage (Steenson et al, 1987) free cells and those on the surface of the beads are not. These cells appear to contribute to the acidification rate, and the presence of bacteriophages slightly reduces the efficiency of the system. Steenson et al. (1987) did not observe any reduced fermentative activity in the presence of phage. This difference might be related to initial cell density since our alginate gels initially contained 10 times more cells than that of Steenson et al. (1987). We must also consider the possibility of phage lysin (virolysin) influence. Accolas and Veaux (1983) suggested that lysin liberated following cell lysis could affect other strains. Since small proteins can diffuse into alginate gels (Tanaka et al., 1984) it is possible that virolysin entered the alginate gel and influenced activity of immobilized bacteria.

CONCLUSION

Since many bacteriophages in milk can survive pasteurization, contamination of an immobilized cell system appears inevitable. Our results have shown that bacteriophages reduce fermentative activity of immobilized mesophilic lactic acid bacteria systems. The use of such systems for continuous inoculation of milk in cheese manufacture would appear even more susceptible to lysis since released cells are not protected from bacteriophages. Although this appears to constitute a major problem, the immobilization of multiple phage unrelated strains might be considered. Thus one strain could be lysed by a given phage while other strains would be unaffected. Since our results suggest that phage resistant cells could multiply in the system (Figure 2) the lysed strain would be replaced by a resistant variant and activity of the culture would increase. Cultures carrying phage would then be frequently produced. Such cultures can be used successfully in cheese manufacture (Stadhouders, 1986). Although activity of these cultures is uneven, complete failure is very rare.

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REFERENCES

ACCOLAS, J.P.; HEMME, D.; DESMAZEAUD, M.J.; VASSAL, L.; BOUILLANNE, C. and VEAUX, M. 1980. Le lait, 60: 487-524.

ACCOLAS, J.P. and VEAUX, M. 1983. La technique laitière #976: 51-59.

BABEL, F.J. 1976. Chap. 6, Dairy Technology and Engineering, AVI Publ. Co., Westport, 631 p.

BERGEYS MANUAL OF DETERMINATIVE BACTERIOLOGY (1986). Vol. II. Williams & Wilkins, Baltimore.

CAVIN, J.F.; SAINT, C. and DIVIES, C. 1985. Biotechnol. Letters, 7(11): 821-826.

CHAMPAGNE, C.P. and BOYAVAL, P. 1986. Technique laitière et marketing #1015: 26-32.

CHAMPAGNE, C.P. and CÔTÉ C.B. 1987. Biotechnol. Letters, 9(5): 329-332.

COGAN, T.M. 1980. Le lait, 60: 397-425.

GILLILAND, S.E. 1985. Bacterial starter cultures for foods. CRC Press, Boca Raton, USA., 205 p.

HUGGINS, A.R. 1984. Food Technol., 38(6): 41

KLAENHAMMER, T.R. 1984. Advances in Appl. Microbiol., 30: 1-27.

LAWRENCE, R.C.; HEAP, H.A.; LIMSOWTIN, G. and JARVIS, A.W. 1978. J. Dairy Sci., 61(8): 1181-1191.

LINKO, P. and LINKO, Y.Y. 1984. CRC critical reviews in biotechnology, 1(4): 289-338.

LINKO, P. 1985. Immobilized lactic acid bacteria. In: Enzymes and immobilized cells in biotechnology, A.I. Laskin (Ed.), Benjamin/Cummings Publ. Co., Mendo Park, Calif., pp 25-36.

OHLSON, S.; LARSON, P.O. and MOSBACH, K. 1979. Eur. J. Appl. Microbiol. Biotechnol., 7: 105-110.

POTTER, N.N. and NELSON, F.E. 1952. J. Bacteriol., 64: 105-111.

PREVOST, H. and DIVIES, C. 1987. Biotechnol. Letters, 9(11): 789-794.

PREVOST, H.; DIVIES, C. and ROUSSEAU, E. 1985. Biotechnol. Letters, 7(4): 247-252.

ROY, D.; GOULET, J. and LEDUY, A. 1987. J. Dairy Sci., 70(3): 506-513.

SHAW, M. 1983. La Technique Laitière, #976(may): 51-59.

STADHOUDERS, J. 1986. Neth. Milk Dairy J., 40(2/3): 155-173.

STEENSON, L.R.; KLAENHAMMER, T.R. and SWAISGOOD, H.E. 1987. J. Dairy Sci. 70(6): 1121-1127.

TANAKA, H., MATSUMURA, M. and VELIKI, I.A. 1984. Biotechnol. Bioeng. 26: 53-58.

TURNER, G.E. and NELSON, F.E. 1951. J. Dairy Sci., 37: 754-762.