Food Microbiology, 1991, 8, 137–142

Use of continuous culture for internal pH determination of lactic acid bacteria

L. C. McDonald, H. M. Hassan†, H. P. Fleming* and M. A. Daeschel‡

Food Fermentation Laboratory, US Department of Agriculture, Agricultural Research Service, and North Carolina Agricultural Research Service, Department of Food Science, North Carolina State University, Raleigh, NC 27695-7624, USA

Received 9 October 1990

The response of cytoplasmic pH to external cellular pH was shown to be influenced by the growth phase for Leuconostoc mesenteroides, *but not for* Lactobacillus plantarum. *A continuous culture system was shown to reduce growth phase effects, and offer the advantage of more consistency in internal pH values. Oxygen, manganese, cell density, external pH, and organic acid production were shown not to influence the internal pH of these bacteria when grown in continuous culture.*

Introduction

The cytoplasmic pH of micro-organisms is becoming of increasing interest to food microbiologists because of its significance in cell physiology and its response to food preservatives. Cessation of growth and metabolism occur at a time when the cytoplasmic pH (pH_{in}) reaches a given value, which is interpreted as the critical value for the internal enzyme activity characteristic of the organism (Kashket 1987).

The external pH (pH_{out}), presence of organic acids, and mode of energy generation (i.e. respiration vs fermentation) influence the bacterial cell's internal pH, (Kashket and Wong, 1969, Kashket 1981a, Baronfsky et al. 1984, Goodwin and Zeikus 1987, Repaske and Adler 1981, Ronning and Frank 1987). It has been suggested that the potency of weak acids as food preservatives is related to their capacity to reduce specifically the intracellular pH (Salmond et al. 1984).

A greater range of internal pH values is exhibited by fermentative than respiratory micro-organisms (Booth 1985). Among the lactic acid bacteria, the limiting cytoplasmic pH has been shown to vary widely from the more acid-sensitive streptococci to the more acid-tolerant lactobacilli (Kashket 1987). Tolerance to low internal pH can be highly significant in the growth sequence of bacterial species in mixed culture fermentations such as with vegetables, where *Leuconostoc mesenteroides* (comparatively low acid tolerance) may initiate the fermentation and *Lactobacillus plantarum* (comparatively high acid tolerance) characteristically terminates

*Corresponding author.
†Present address: Department of Food Science, NC State University, Raleigh, NC 27695-7624, USA.
‡Present Address: Department of Food Science and Technology, Oregon State University, Corvallis, OR 97331, USA.

Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the US Department of Agriculture or North Carolina Agricultural Research Service, nor does it imply approval to the exclusion of other products that may be suitable.

the fermentation (McDonald et al. 1990). While each of these bacteria was shown to have a specific limiting internal pH, the limiting external pH varied with the growth medium.

Thus, measurement of cytoplasmic pH may be essential for further understanding the mechanism of acid tolerance among micro-organisms, including the lactic acid bacteria.

Most studies on the cytoplasmic pH of cells have been done with batch cultures, even though the pH may be influenced by the cell's growth phase (Kashket 1981a,b, Baronofsky et al. 1984). Micro-organisms grown in batch culture exhibit multiple growth phases (i.e. lag, exponential, stationary and death) (Drew 1981), while cells grown in continuous culture are maintained in the exponential growth phase (Monod 1950, Ingraham et al. 1983). Growth of micro-organisms is influenced by environmental factors including temperature, pH, available nutrients, and oxygen.

In this study, we show the merits of growing cells of lactic acid bacteria in continuous rather than in batch culture when wishing to determine the cytoplasmic pH and its responses to extracelluar environments.

Materials and Methods

Cultures and media

Lactobacillus plantarum WSO (from the US Food Fermentation Laboratory, Raleigh, NC) and *Leuconostoc mesenteroides* C 33 (from J. R. Stamer, Cornell University, Geneva, NY) were maintained by weekly transfers into MRS broth at 22°C. The growth medium, modified HHD (MHHD) (McDonald et al. 1987), was composed of trypticase peptone (10 g^{-1}; BBL Microbiology Systems, Cockeysville, MD), casamino acids (3 g^{-1}; Difco Laboratories, Detroit, MI), phytone peptone (1·5 g^{-1}; BBL), yeast extract (1 g^{-1}; BBL), and Tween 80 (1 g^{-1}; Fisher Scientific Company, Fairlawn, NJ). MHHD was adjusted to pH 7·0 and autoclaved for 15 min. Glucose (Sigma Chemical Company, St. Louis, MO) and $MnSO_4 \cdot H_2O$ (Fisher) solutions were autoclaved separately and added to MHHD after autoclaving. The final glucose concentration in the medium was 2% (111mM). When used in the study, the final concentration of manganese was 10 mM.

Inocula were prepared as follows. Each micro-organism was transferred from MRS broth into MHHD and incubated for 18 h at 30°C. A 1% inoculum from the 18-h culture was again transferred to MHHD and incubated for 18 h at 30°C. This 18-h culture was used as inoculum for batch culture or continuous culture.

Batch culture

For batch culture studies, a 1% inoculum was added to MHHD and incubated at 30°C. Growth was followed at an absorbency of 650nm (OD_{650}) using a Lumetron Colorimeter (Photovolt Corporation, Indianapolis, IN). For pH_{in} measurements, samples were taken at mid-log phase and at stationary phase. Growth phases were determined from growth curve studies.

Continuous culture

The continuous culture system consisted of a reservoir containing 8 l of MHHD, a Gilson Minipuls 2 pump, a 1·5-l Multigen growth vessel (New Brunswick Scientific Company, Inc., New Brunswick, NJ), and an overflow receptacle. The growth vessel contained 600 ml growth medium and was maintained at 30°C. Mixing of cells and incoming fresh medium was achieved by agitation at 140 ± 20 rpm (knob setting at 1·8). An air vent with a 0·45-μm pore size filter was left open at the top of the growth vessel. Where indicated, sterile oxygen (100%) was introduced into the growth vessel through the air vent at a flow rate of 50 ml min^{-1}. A 1% inoculum was added to the growth vessel and allowed to grow as a batch culture until late log phase before fresh medium was pumped into the growth vessel. The dilution rate was adjusted to a rate less than the maximum specific growth rate of the culture in MHHD (0·12 h^{-1} for *L. plantarum* and 0·16 for *Leuconostoc mesenteroides*) in order to avoid washout (Ingraham et al. 1983). Optical density was monitored at 650 nm. When steady state was established (i.e. stable OD_{650} for 10–12 generations), samples were taken for internal pH measurements. Before each assay, cells were examined microscopically to ensure that the culture was not contaminated.

Internal pH and cell volume

The internal pH and cell volume were determined using radioactive probes (isotypes from Dupont Company, Wilmington, DE), as described by Rottenberg (1979) and adapted by McDonald et al. (1990). For internal pH measurement, [U-^{14}C]salicylic acid was used. For cell volume determination, [U-^{14}C]sorbitol and $^{3}H_2O$ were used.

For internal pH measurements with non-growing cells, the cells were centrifuged at 22°C at 9000 *g* for 10 min, washed in MHHD, and resuspended to an OD_{650} of 1·0 in MHHD adjusted to the desired pH (with HCl). The desired radioisotope was then added to this cell suspension. For growing cells, an aliquot was removed from the continuous culture, and the *isotope* was added directly to this cell suspension.

Analytical procedures

The concentrations of lactic and acetic acids were determined by using high pressure liquid chromatography (McFeeters et al. 1984).

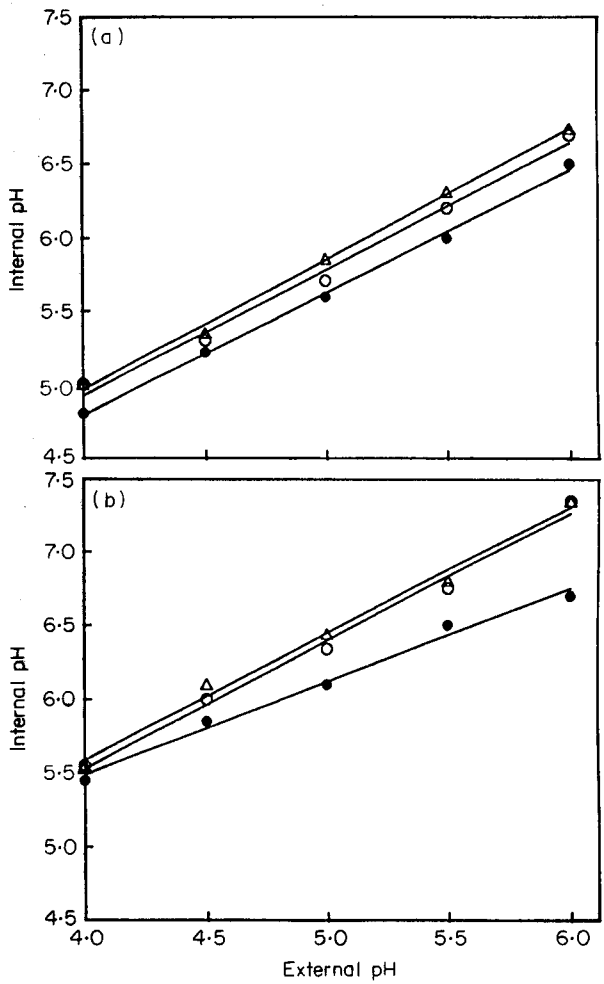

Fig. 1. Effect of growth phase and pH_{out} on response of pH_{in} of *L. plantarum* (a) and *Leuconostoc mesenteroides* (b). Internal pH values plotted are the -Log_{10} of the mean of the H^+ concentration from duplicate assays. Linear regression lines are drawn. All lines have r^2 >0·98. Exponential growth phases, batch (o) and continuous (Δ), and the stationary growth phase (•) are shown in both (a) and (b). Slopes of the regression lines are as follows; (a) batch 0·89, continuous 0·86, stationary 0·88; (b) batch 0·86, continuous 0·87, stationary 0·63.

Acids were analysed with an Aminex HPX-87H column (Bio-Rad Laboratories, Richmond, CA) with a cation guard column. The solvent was 0·01 N H_2SO_4. A refractive index detector was used for quantitation of the acids. The external cellular pH was determined with a Fisher model 855MP Accumet pH meter. The optical density (650 nm) was determined with a Lumetron Colorimeter.

Results and Discussion

Figure 1 shows that the pH_{in} response of both *L. plantarum* and *Leuconostoc mesenteroides* was dependent upon pH_{out} in washed cells. As pH_{out} decreased, pH_{in} decreased. For both *L. plantarum* and *Leuconostoc mesenteroides*, a larger pH differential between pH_{in} and PH_{out} could be maintained with cells in the exponential phase than with cells in the stationary phase.

Regression and covariance analysis were used to evaluate the pH response of cells in terms of statistically significant differences. Because it was unclear whether the evaluation should be based upon hydrogen ion concentration ($[H^+]$) or pH, we first evaluated the data directly in terms of $[H^+]$ and then transformed as pH. The equality of variances about the fitted regression lines over the entire range of data was examined for both $[H^+]$ and pH. These variances were quite similar for data expressed as pH, but quite dissimilar for data expressed as $[H^+]$, thus supporting application of standard statistical techniques (i.e. those that assume stable variances) for data expressed as pH. We then tested whether separate similar regressions of pH_{in} against pH_{out} adequately fit each of six cases shown in Fig. 1. Significant deviation from linear response for pH_{in} vs pH_{out} was not found ($P>0.05$). Therefore, it was deemed appropriate to evaluate treatment differences based on slopes of pH.

Exponential (either batch or continuous culture) cells of *Leuconostoc mesenteroides* had a slope of about 0·87 (Fig. 1); stationary phase cells of *Leuconostoc mesenteroides* had a slope estimate of 0·63 which differs significantly ($P\geq0.05$) from 0·87. The difference between slopes of *L. plantarum* was not significant, with

Table 1. Influence of oxygen, manganese, cell density, organic acid production, and external pH on the internal pH of *L. plantarum* and *Leuconostoc mesenteroides* grown in continuous culture[a]

Growth conditions[b]		Cell density	Lactic acid (mM)	Acetic acid (mM)	External pH	Internal pH
O_2	Mn^{2+}					
L. plantarum						
-	-	0·25	13·81	0·91	4·87	5·93
+	-	0·18	11·41	1·01	5·20	5·90
-	+	3·30	55·23	7·02	3·77	5·90
+	+	3·48	61·46	7·22	3·71	5·88
Leuconostoc mesenteroides						
-	-	0·58	8·00	1·78	4·85	6·45
+	-	0·63	8·30	2·14	5·00	6·43
-	+	0·83	12·00	2·01	4·80	6·41
+	+	0·87	12·13	1·88	4·81	6·50

[a] Cell density (OD_{650}), acids, and pH were determined at steady state conditions.
[b] The basal medium was MHHD, pH 7·0. See Materials and Methods for details.
Dilution rates for medium addition were 0·055 h^{-1} for *L. plantarum* and 0·08 h^{-1} for *Leuconostoc mesenteroides*.

exponential (batch or continuous culture) and stationary phase cells having slopes of about 0·88. Internal pH differences between exponential and stationary phase cells have been found in other studies (Kashket 1981a,b, Baronofsky et al. 1984).

Introduction of manganese to a continuous culture of *L. plantarum* resulted in pronounced changes in cell density, lactic acid production and external pH (Table 1). Much smaller effects were seen with *Leuconostoc mesenteroides*. Manganese is required for biosynthetic functions and has been shown to exert a protective effect against oxygen toxicity in certain lactic acid bacteria, including *L. plantarum* and *Leuconostoc mesenteroides* (Archibald and Fridovich 1981, Raccach 1985, Raccach and Marshall 1985). Oxygen has been shown to be toxic and, most living cells possess superoxide dismutases, which protect against toxicity (Hassan and Fridovitch 1980). The pH_{in} values of *L. plantarum* and *Leuconostic mesenteroides* were 5·80–5·90 and 6·4–6·5, respectively, regardless of the presence or absence of manganese or oxygen. This suggests that alteration in environment through addition of manganese or oxygen did not affect the cell's ability to control pH_{in} as long as the cell's growth remained in the exponential phase. However, this would not be the case for all organisms. Microorganisms which contain both respiratory and fermentative pathways maintain higher pH_{in} when respiring (Padan et al. 1976, Kashket 1981b, Michels and Bakker 1985). Therefore, the pH_{in} of an organism capable of both respiration and fermentation would depend on the presence of oxygen in the cell's environment.

The results show that growth phase may influence a cell's ability to control pH_{in}. However, no difference in pH_{in} response was found between exponential phase cells, whether grown in batch or continuous culture. This suggests that continuous culture data can be compared with log phase batch culture data. Use of a continuous culture system should eliminate differences due to growth phase, provide a continuous supply of cells for pH_{in} studies, and yield reproducible results. Furthermore, the continuous culture system is simpler to use in that it eliminates the continual monitoring of cell growth to ensure log phase required for batch culture studies. It also allows the investigator to change and control the specific growth rate of an organism in a given environment simply by changing the dilution rate.

Acknowledgements

This investigation was supported in part by a research grant from Pickle Packers International, Inc., St. Charles, IL. The authors thank Dr K. P. Burnham, USDA-ARS and Department of Statistics, North Carolina State University, for advice and assistance in the statistical analysis of pH data.

References

Archibald, F. S. and Fridovich, I. (1981) Manganese, superoxide dismutase, and oxygen tolerance in some lactic acid bacteria. *J. Bacteriol.* **146**, 928–936.

Baronofsky, J. J., Schreurs, W. J. A. and Kashket, E. R. (1984) Uncoupling by acetic acid limits growth of and acetogenesis by *Clostridium thermoaceticum*. *Appl. Environ. Microbiol.* **48**, 1134–1139.

Booth, I. R. (1985) Regulation of cytoplasmic pH in bacteria. *Microbiol Rev.* **49**, 359–378.

Drew, S. W. (1981) Liquid culture. In *Manual of Methods for General Bacteriology* (Eds Gerdhart, P., Murray, R. G. E., Costilow, R. N., Nester, E. W., Wood, W. A., Krieg, N. R. and Briggs, G.) pp. 151–178. Washington, American Society for Microbiology.

Goodwin, S. and Zeikus, J. G. (1987) Physiological adaptations of anaerobic bacteria to low pH: metabolic control of proton motive force in *Sarcina Ventriculi*. *J. Bacteriol*, **169**, 2150–2157.

Hassan, H. M. and Fridovitch, I. (1980) Superoxide dismutases: detoxication of a free radical. In *Enzymatic Basis of Detoxication* (Ed. Jakoby, W. B.) Vol. 1, pp. 311–332. New York, Academic Press.

Ingraham, J. L., Maaloe, O. and Neidhardt, F. C. (1983) *Growth of the Bacterial Cell*. pp. 240–245. Sunderland, MA, Sinauer Associates, Inc.

Kashket, E. R. (1981a) Proton motive force in growing *Streptococcus lactis* and *Staphylococcus aureus* cells under aerobic and anaerobic conditions. *J. Bacteriol*. **146**, 369–376.

Kashket, E. R. (1981b) Effects of aerobiosis and nitrogen source on the proton motive force in growing *Escherichia coli* and *Klebsiella pneumoniae* cells. *J. Bacteriol*. **146**, 377–384.

Kashket, E. R. (1987) Bioenergetics of lactic acid bacteria: cytoplasmic pH and osmotolerance. *FEMS Microbiol. Rev*. **46**, 233–244.

Kashket, K. R and Wong, T. S. (1969) The intracellular pH of *Escherichia coli*. *Biochim. Biophys. Acta* **193**, 212–214.

McDonald, L. C., McFeeters, R. F., Daeschel, M. A. and Fleming, H. P. (1987) A differential medium for the enumeration of homofermentative and heterofermentative lactic acid bacteria. *Appl. Environ. Microbiol*. **53**, 1382–1384.

McDonald, L. C., Fleming, H. P. and Hassan, H. M. (1990) Acid tolerance of *Leuconostoc mesenteroides* and *Lactobacillus plantarum*. *Appl. Environ. Microbiol*. **56**, 2120–2124.

McFeeters, R. F., Thompson, R. L. and Fleming, H. P. (1984) Liquid chromatographic analysis of sugars, acids, and ethanol in lactic acid vegetable fermentations. *J. Assoc. Off. Anal. Chem*. **67**, 710–714.

Michels, M. and Bakker, E. P. (1985) Generation of a large protonophore-sensitive proton motive force and pH difference in the acidophilic bacteria *Thermoplasma acidophilum* and *Bacillus acidophilus*. *J. Bacteriol*. **161**, 231–237.

Monod, J. (1950) La technique de culture continue. Theorie et applications. *Ann. L'Inst. Pasteur*, Paris **79**, 390–410.

Padan, E., Zilberstein, Z. and Rottenberg, H. (1976) The proton electrochemical gradient in *Escherichia coli* cells. *Eur. J. Biochem*. **63**, 533–541.

Raccach, M. (1985) Manganese and lactic acid bacteria. *J. Food Protect*. **48**, 895–898.

Raccach, M. and Marshall, P. S. (1985) Effect of manganese ions on the fermentative activity of frozen-thawed lactobacilli. *J. Food Sci*. **50**, 665–668.

Repaske, D. R. and Adler, J. (1981) Change in intracellular pH of Escherichia coli mediates the chemostatic response to certain attractants and repellents. *J. Bacteriol*. **145**, 1196–1208.

Ronning, I. E. and Frank, H. A. (1987) Growth inhibition of putrefactive anaerobe 3679 caused by stringent-type response induced by protonphoric activity of sorbic acid. *Appl. Environ. Microbiol*. **53**, 10220-10227.

Rottenberg, H. (1979) The measurement of membrane potential and pH in cells, organelles, and vesicles. *Methods Enzymol*. **50**, 547.

Salmond, C. V., Kroll, R. G. and Booth, I. R. (1984) The effect of food preservatives on pH homeostasis in *Escherichia coli*. *J. Gen. Microbiol*. **130**, 2845–2850.

Food Microbiology, 1991, **8**, 355

Erratum

Use of continuous culture for internal pH determination of lactic acid bacteria

L. C. McDonald, H. M. Hassan, H. P. Fleming and M. A. Daeschel

Food Microbiology, 1991, **8**, 137–142

In the Materials and Methods section on page 138, the growth medium components of modified HHD (MHHD) should have been expressed as $\mathbf{gl^{-1}}$ instead of g^{-1}.