APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1988, p. 1627–1629 Vol. 54, No. 6
0099-2240/88/061627-03$02.00/0

A pH Control System Based on Malate Decarboxylation for the Cultivation of Lactic Acid Bacteria†

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Received 7 January 1988/Accepted 6 March 1988

Most species of lactic acid bacteria decarboxylate L-malate to lactate and CO_2 if an energy source such as glucose is present. A proton is taken up in the reaction, which prevents pH decreases in the growth medium caused by lactic acid production from glucose fermentation. MRS broth (pH 7.0) (Difco Laboratories) containing 10 mM glucose and various concentrations of L-malate (0, 25, 50, 75, and 100 mM) was used to cultivate *Lactobacillus plantarum*. After 72 h at 37°C, all malate was decarboxylated and all glucose was fermented, with resultant final pH values of 4.5, 6.3, 6.9, 7.3, and 7.5, respectively. When D-malate (which cannot be decarboxylated) was substituted for L-malate, the final pH values were 4.5, 5.2, 5.6, 5.8, and 5.9. By varying the ratios of glucose to L-malate in the growth medium, it was possible to obtain pH values which were lower, the same, or higher than the initial pH values. In contrast, buffers such as phosphate only retard decreases in pH. L-Malate, when compared with K_2PO_4 on an equal molar basis, provided greater resistance to decreases in pH. Higher specific growth rates were observed for *L. plantarum* and *Leuconostoc mesenteroides* when L-malate rather than K_2PO_4 was incorporated into the growth medium.

The decarboxylation of L-malate to lactate and CO_2 by malolactic enzyme (EC 1.1.1.38) is a catabolic activity found only with lactic acid bacteria. The malolactic reaction is novel in that energy is not generated, but the reaction appears to be beneficial to cells because of an increased growth rate. The literature suggests (5) that the significance of the malolactic enzyme is that it compensates for the hydrogen ions produced during carbohydrate fermentation by reducing the acidity of the medium through the conversion of a dicarboxylic acid (malate) to a monocarboxylic acid (lactate), with a resultant rise in pH. Recently, Henick-Kling (T. Henick-Kling, Ph.D. thesis, University of Adelaide, Adelaide, Australia, 1986) proposed a hypothesis which complements the above-described hypothesis by suggesting that the decarboxylation of malate gives an energetic advantage by increasing the internal pH of the cell. The alkalizing effect of malate decarboxylation was utilized in designing a differential medium (2) to distinguish between malate-decarboxylating and non-decarboxylating strains of lactic acid bacteria. It was noted (M. A. Daeschel, R. F. McFeeters, H. P. Fleming, T. R. Klaenhammer, and R. B. Sanozky, U.S. patent 4,666,849, May 1987) that by changing the ratios of glucose to L-malate, the pH values in fermented media could actually be increased up to 8.58 from an initial value of 7.00, depending on the strain of lactic acid bacterium. These observations formed the basis of the present study. Specific objectives of the study were to (i) evaluate the performance of L-malate as a pH control agent in media for the cultivation of lactic acid bacteria, (ii) compare L-malate decarboxylation with a phosphate-buffered system as means of pH control, and (iii) evaluate the effects of L-malate decarboxylation on the growth rate of lactic acid bacteria as compared with the effects of a phosphate-buffered system.

The strains of bacteria used, *Lactobacillus plantarum* WSO and *Leuconostoc mesenteroides* LC-33, were maintained in MRS broth (Difco Laboratories). A modified MRS medium was used in experiments designed to evaluate the buffering capacity of decarboxylated malate. The modification consisted of omitting ammonium citrate, sodium acetate, and disodium phosphate, which are the principal buffering components of MRS broth. Glucose was also omitted.

K_2PO_4, L-malate, D-malate, and D-glucose were added to the modified MRS medium at the indicated concentrations. The pH of the medium was adjusted to 7 before autoclaving. Relative comparisons of growth in various medium formulations were made by determining the specific growth rate constants (k), which have the dimension of reciprocal hours. The constant k was experimentally determined by plotting the $\log_{10}$ of optical density readings versus time. The slope of the linear portion of the growth curve is equal to $k/(2.303)$ (3). Glucose concentrations were determined by the colorimetric method of Sumner and Sisler (8). Lactic and malic acids were measured by using high-performance liquid chromatography according to the procedures of McFeeters et al. (6).

The effect of L-malate decarboxylation on the pH of the growth medium is shown in Fig. 1. D-Malate, which cannot serve as a substrate for the malolactic enzyme, will function as a weak buffer (pK_1, 3.8; pK_2, 5.11). This ability was seen (Fig. 1) as a pH differential of ca. 1.5 pH units when 100 mM D-malate was added, as compared with no D-malate added. However, when 100 mM L-malate was added, a pH differential of ca. 3.0 U was noted. The difference between the pH values obtained when D- and L-malate were added is presumed to be due to the decarboxylation of L-malate. Analysis of the growth medium after 72 h showed that in each case all glucose was fermented to lactic acid and all L-malate was catabolized to lactate, whereas D-malate remained unchanged. The pH values of the medium were also determined after 72 h and are indicated as final pH. It should be noted that when the concentration of added L-malate exceeded 50 mM, the final pH went above the initial pH of 7.

Phosphate salts are commonly used as buffers in microbi-

† Paper no. 11345 of the Journal Series of the North Carolina Agricultural Research Service.

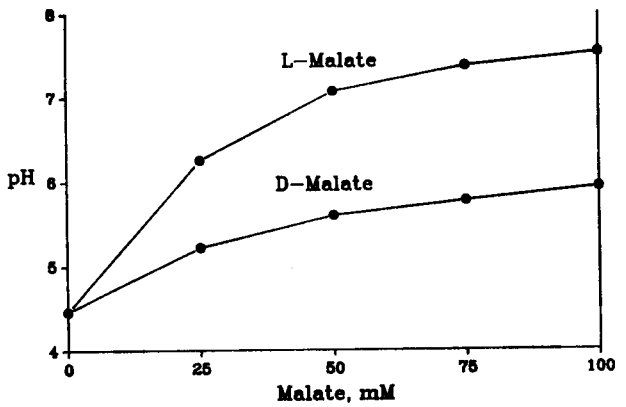

FIG. 1. Effect of the concentration of D- or L-malate in modified MRS broth on the final pH obtained when 10 mM glucose was completely fermented by *L. plantarum* at 37°C. In each treatment, L-malate, when added, was completely decarboxylated, whereas D-malate remained unchanged.

ological media; hence, a comparison of K_2HPO_4 with D- and L-malate as pH control agents was made (Fig. 2). An additional experimental variable was varying the ratio of glucose to KH_2PO_4, L-malate, or D-malate. In all cases L-malate provided a greater resistance to pH change than did either K_2HPO_4 or D-malate. D-Malate provided better buffering than K_2HPO_4 when the ratio of glucose to buffer was greater than 3.7. At ratios lower than 3.7, K_2HPO_4 gave better buffering. This observation may reflect the difference in pK values for D-malate (3.8 and 5.11) and phosphate (7.2) within the region of these pH changes.

Table 1 shows the effects of incorporation of D-malate, L-malate, or K_2HPO_4 on the growth rate of *L. plantarum* and *Leuconostoc mesenteroides* in modified MRS broth. With both species, a slower growth rate was observed with K_2HPO_4 than without it (glucose only) or than with L-malate or D-malate. There did not appear to be any significant differences between D- and L-malate in their effects on the growth of *L. plantarum*; however, L-malate appeared to be

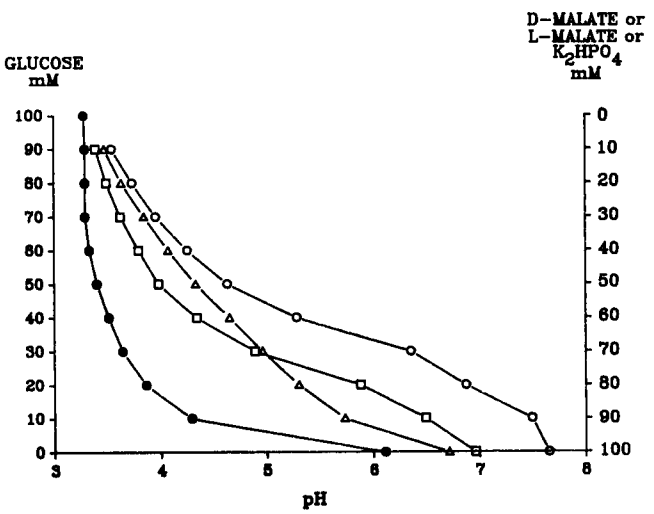

FIG. 2. Effect of different ratios of glucose and L-malate (○), glucose and D-malate (△), or glucose and K_2HPO_4 (□) in modified MRS broth on the final pH when fermented by *L. plantarum* at 37°C. ●, Glucose only.

TABLE 1. Effect of 50 mM L-malate, D-malate, or K_2HPO_4 on the specific growth rate of *L. plantarum* and *Leuconostoc mesenteroides* in modified MRS medium containing 50 mM glucose

Strain	Specific growth rate constant k (h^{-1})[a] with:			
	L-Malate	D-Malate	K_2HPO_4	Glucose only
Lactobacillus plantarum WSO	0.92	0.92	0.66	0.82
Leuconostoc mesenteroides LC-33	0.44	0.38	0.29	0.44

[a] Values are means of duplicate determinations.

slightly stimulatory for *Leuconostoc mesenteroides* as compared with D-malate. Both D- and L-malate were stimulatory for *L. plantarum* as compared with glucose alone.

Decarboxylation of L-malate (malate + H^+ → lactate + CO_2) can, by the uptake of H^+, neutralize the acidity from sugar fermentation. The degree of neutralization was shown to be dependent on the relative proportions of L-malate and sugar. Thus, decarboxylation of L-malate can provide a means of resistance to pH change, which in effect serves the same function as a buffer. However, L-malate decarboxylation cannot be regarded as a true buffer, since it is not governed by an ionization constant. Although malate (itself a weak acid) is a buffer, this effect is secondary, as shown in the experiments with D-malate, which is not decarboxylated. True buffers simply provide resistance to pH change, whereas L-malate decarboxylation can result in an increase in pH in an environment where acid is being produced. This feature may provide a basis for unique applications.

Another mechanism by which some lactic acid bacteria can alkalize their surrounding environment is through the arginine deimidase pathway, which catalyzes the conversion of arginine to ornthine, carbon dioxide, and ammonia (1). This pathway has been demonstrated for *L. plantarum* (4), with which it was observed that medium containing 15 mM arginine and 2.8 mM glucose with an initial pH reaction of 6.0 rose to pH 8.6 after growth. Whether this mechanism can be utilized as a pH control system for cultivating lactic acid bacteria is a question that may warrant investigation.

Various types of neutralizing agents and methods are used in the cultivation of lactic acid bacteria for the industrial production of starter culture concentrates, the propagation of bulk starter, and the production of lactic acid. Buffers commonly used in growth media include organic and inorganic phosphates, calcium carbonate, ammonium hydroxide, calcium hydroxide, sodium hydroxide, and the salts of organic acids such as acetate. These buffers are applied either as a part of an external pH control system, where the agent is fed into the growth medium on demand, or as part of an internal pH control system that relies on the solubilization of buffers in the growth medium in response to the culture producing acid in the growth medium (W. E. Sandine and J. W. Ayres, U.S. patent 4,282,255, Aug. 1981). Dialysis of acid out of the growth medium is still another method of pH control.

All of the above-mentioned methods and agents have disadvantages, which include precipitation, chelation, cation damage to cells, and decreased growth rates of cells. The use of L-malate as a neutralizing agent in media for the cultivation of lactic acid bacteria may be practical and is worthy of further investigation. The data presented in this study indi-

cate that L-malate decarboxylation is superior to phosphate as a neutralizing agent and does not cause the decrease in growth rate observed with phosphate. The decarboxylation of L-malate generates CO_2 in the medium, as well as additional lactate. It is not known whether this will present disadvantages. It has been observed (7) that carbon dioxide in growth media can benefit the growth of certain lactobacilli.

This investigation was supported in part by a research grant from Pickle Packers International, Inc., St. Charles, Ill.

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