Malic Acid as a Source of Carbon Dioxide in Cucumber Juice Fermentations

R. F. McFEETERS, H. P. FLEMING, and R. L. THOMPSON

ABSTRACT

The degradation of malic acid to lactic acid and CO_2 during fermentation of cucumber juice was investigated. This malolactic reaction was the major source of CO_2 when cucumber juice was fermented by *Lactobacillus plantarum*. It may also be an important CO_2 source in controlled cucumber fermentations. In addition to CO_2 production, the degradation of malate served to buffer the fermentation and increase sugar utilization. The pH after 7 days' fermentation was 2.8 when 13 mM malic acid was present in the juice and 4.1 with 135 mM malic acid. In the same fermentations, 52% of the sugars were degraded with the low malic acid concentration while complete sugar utilization was observed with the highest malic acid level.

INTRODUCTION

BLOATING of brined cucumbers has been attributed to CO_2 production by gas-forming yeasts and bacteria present during fermentation (Etchells and Bell, 1950; Etchells et al., 1968). However, Fleming et al. (1973a) found that considerable amounts of CO_2 were produced when cucumbers were fermented with a pure culture of *Lactobacillus plantarum*, a homofermentative lactic acid bacterium that is generally considered to be a nongas-former. The CO_2 produced by *L. plantarum* and by the fruit was sufficient to result in serious bloater formation (Fleming et al., 1973b). The reactions responsible for CO_2 production were not determined.

Hirose (1976) reported that malic acid is the major organic acid in Japanese fresh market cucumbers. McFeeters et al. (1982) found that malic acid is also the principal organic acid in pickling cucumbers. It is known that many lactic acid bacteria have an inducible malo-lactic enzyme which degrades malate to lactate and CO_2 (Kunkee, 1967; Radler, 1966; Schutz and Radler, 1973). This has been found to be an important reaction in decreasing the acidity of wines (Kunkee, 1967; Beelman and Gallander, 1979). Radler (1975) showed that *Leuconostoc mesenteroides* quantitatively degraded malic acid to lactic acid and CO_2 without significantly affecting the pathway of glucose fermentation by this heterolactic organism.

The objectives of this paper were to determine whether malic acid degradation was a major source of CO_2 production during fermentation of cucumber juice by *L. plantarum*, and to study the effect that the malo-lactic reaction might have on pH and sugar utilization during juice fermentation.

MATERIALS & METHODS

CUCUMBER JUICE was prepared from 4.5–5.7 cm diameter 'Chipper' fruit. Fresh cucumbers were cut into chunks and frozen. They were then thawed and the juice removed using a wine press. Ten liter lots of the juice were heated to the boiling point. After cooling, the juice was frozen until use.

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L. plantarum (WSO) was used in all experiments. The organism was grown at 30°C in MRS broth (De Man et al., 1960). When NaCl was added to cucumber juice in an experiment, 4% NaCl was added to the MRS broth. Cells were harvested 12–15 hr after inoculation into MRS. They were pelleted by centrifugation for 10 min at 12,000 X *g*, washed once with sterile saline, resuspended in an equal volume of cold cucumber juice, and then diluted 15-fold with cold cucumber juice to give an absorbance at 650 nm of 0.07–0.1 prior to inoculation.

Cucumber juice was thawed and filtered through a 0.22 μm, sterile, Millipore GS filter (Millipore Corp., Bedford, MA) attached to a 10-ml disposable syringe into a 15-ml sterile vacutainer (Becton-Dickinson, Rutherford, NJ) tube. Usually 9.0 ± 0.1 ml of juice was put into each vacutainer. To obtain this accuracy, 9.0 ml of juice was pipetted into the filter syringe. The tube was weighed to the nearest 0.1g on a top-loading balance before and after addition of cucumber juice to check the accuracy of filling.

To obtain uniform initiation of fermentation, the sterile juice tubes were kept at 4°C and the cells were resuspended and diluted into 4°C cucumber juice. A 1.0 ml inoculum of diluted cells was put into the vacutainer tubes with a 1.0 ml tuberculin syringe. The fermentation was started by placing all tubes in an experiment into a 30°C water bath. To determine fermentation characteristics in the presence of NaCl, 6% NaCl and various concentrations of malic were added to cucumber juice. The juice was diluted 15% to allow for these additions, and the pH of the samples was adjusted to 5.7. The samples were fermented for 7 days at 30°C.

Turbidity measurements were made directly on the inoculated tubes with a Lumetron colorimeter using a 650 nm filter. Total CO_2 produced in the tubes at specified times was determined by injecting 1.0 ml of 2.0N CO_2-free NaOH solution into the tube containing 10 ml of juice. This raised the pH above 7.0, thus dissolving headspace CO_2. The tubes were shaken well to assure complete absorption of CO_2. A measured sample was then removed from the vacutainer tube with a 10-ml syringe and analyzed for CO_2 by the procedure of Fleming et al. (1974). An Orion model 901 pH meter (Orion Research Inc., Cambridge, MA) was used for pH measurements. Total reducing sugar was measured using the dinitrosalicylic acid reagent (Sumner and Sisler, 1944). L-Lactic acid was measured enzymatically by reduction of NAD to NADH using lactate dehydrygenase (Sigma Chemical Co. Technical Bulletin No. 826-UV). Total lactic acid and malic acid were measured using HPLC with a 5 μm, 8 mm, C_{18} Radial-Pak cartridge from Waters (Waters Associates, Milford, MA). The eluant was pH 2.5, 0.05M ammonium phosphate buffer. Individual sugars were analyzed by HPLC using a Bio-Rad HPX-87HM, Pb-treated anion exchange column (Bio-Rad Labs, Richmond, CA) with water as an eluant. A Waters 6000A pump and model 401 refractive index detector was used. Concentrations were estimated based upon peak height measurements with external standards using a Spectra-Physics 4100 computing integrator (Spectra-Physics Autolab Div., San Jose, CA).

In the experiments, five tubes were used at each sampling point. Turbidity determinations were made on each tube. Three tubes were used for CO_2 measurement. The other two tubes were used for sugar, pH and organic acid analysis. In the figures each point is the mean of these determinations.

RESULTS & DISCUSSION

THE TIME COURSE of malic acid disappearance during cucumber juice fermentation by *L. plantarum* was compared to the growth of the culture, production of CO_2 and formation of L-lactic acid (Fig. 1). Several important points should be noted in this experiment. First, the malic acid was removed completely from the cucumber juice within 7 hr. Secondly, CO_2 production closely paralleled

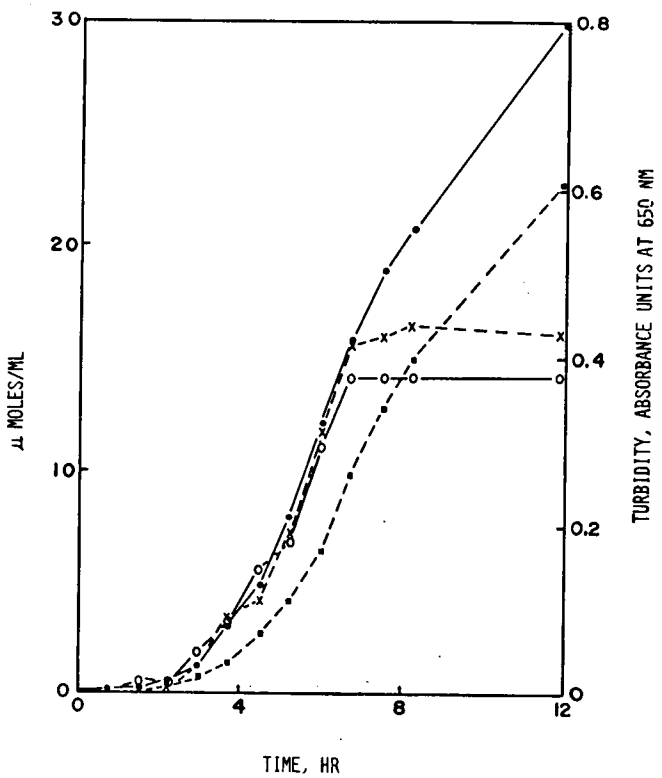


Fig. 1—Time course of cucumber juice fermentation with L. plantarum. Turbidity, --■--; malic acid disappearance, —○—; CO_2 formation, --x--; L-lactic acid formation, —●—.

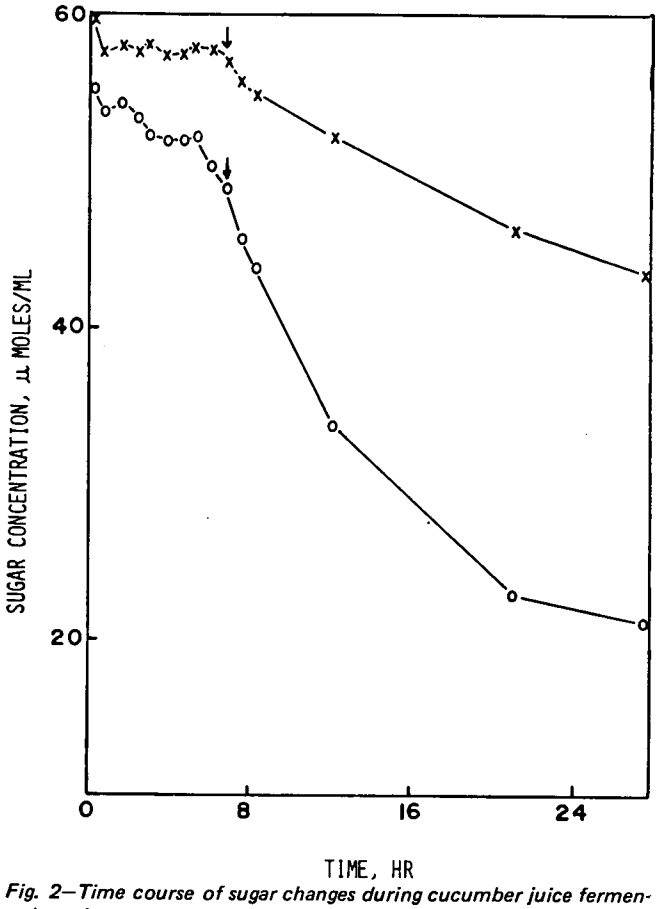


Fig. 2—Time course of sugar changes during cucumber juice fermentation. Arrows indicate the last point at which malic acid was detected. Fructose, x; glucose, o.

malic acid disappearance, even though on a molar basis the CO_2 production slightly exceeded the malate metabolized. Assuming production of 1 mole of CO_2 per mole of malic acid, approximately 85% of the CO_2 produced would have been derived from malic acid. Thirdly, L-lactic acid, the other product of the malo-lactic reaction, also increased in parallel with growth and with malic acid disappearance. *L. plantarum* produces DL-lactic acid by the glycolytic pathway, however, so the L-lactic acid continues to increase after malic acid is gone. Although the initial L-lactic production was consistent with malate degradation by a malo-lactic enzyme, it is probable that some of the L-lactic acid was produced by the glycolytic pathway.

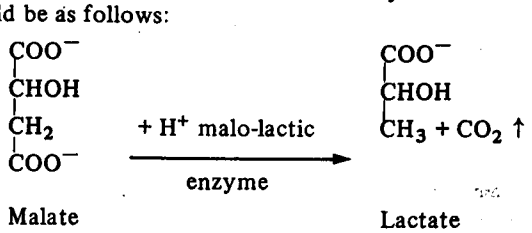
Fig. 2 shows the sugar disappearance during the experiment shown in Fig. 1. The arrows indicate the time at which malic acid could no longer be detected. Neither glucose nor fructose began to be degraded rapidly until malic acid was nearly gone. The cells used to initiate the fermentation were grown in MRS broth without malic acid and so should not have had the malo-lactic enzyme at the time of inoculation. It should also be noted that glucose was utilized more rapidly in the fermentation than fructose. This same pattern of sugar use was observed by Marsali et al. (1977).

Since malic acid is the major organic acid in pickling cucumbers (McFeeters et al., 1982), juice could not be obtained lacking malic acid. Therefore, it was not possible to show that the CO_2 and L-lactic production failed to occur in the absence of malic acid. However, it was possible that the parallel changes in malic acid, L-lactic acid and CO_2 were a coincidental occurrence rather than a direct substrate/product relationship. To eliminate this possibility, the previous time course experiment was repeated with cucumber juice to which additional L-malic acid was added. The pH of the malic acid supplemented juice was adjusted to pH 5.7, the original pH of the juice.

Fig. 3 shows that when more malic acid was utilized, CO_2 production again paralleled malic acid disappearance and stopped when malic acid was gone. The increase in total CO_2 was nearly equal to the increase in malic acid consumed. Not shown in Fig. 3, L-lactic acid production closely paralleled malic acid disappearance, but again the L-lactic continued to be formed after malic acid was gone. These results confirm that malic acid degradation is the major CO_2 source in cucumber juice fermentation.

Kandler et al. (1973) concluded that the malo-lactic reaction is not a source of energy for bacteria since the molar growth yield of *Leu. mesenteroides* grown on glucose was not affected by the addition of malic acid. Radler (1966) proposed that the malo-lactic reaction buffers fermentation and allows organisms to utilize more substrate and grow for longer periods of time. Support for this idea came from the observations that *L. casei* could use more sugar in pH 3.7 medium with malic acid present (Radler, 1966) and the pH of *Leu. mesenteroides* fermentations remained higher with malic acid added (Kandler et al., 1973).

The pH of pickling cucumbers is normally in the range 5.5–6.0 (McFeeters et al., 1982). In this pH range, malic acid will exist primarily as the dianion. The net result of the malo-lactic reaction in a cucumber juice fermentation should be as follows:



There will be uptake of one proton per malate degraded. The product of the fermentation in addition to CO_2 will

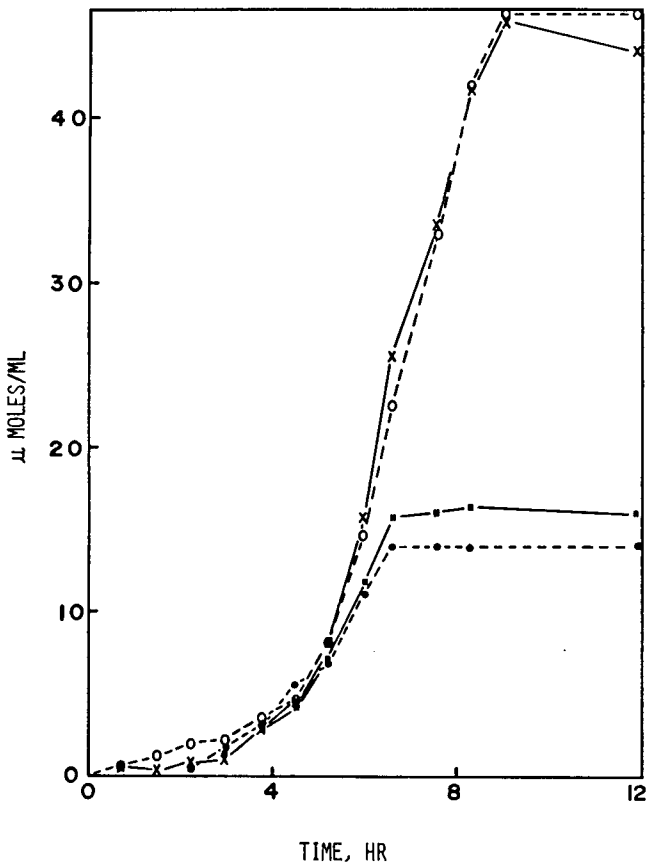


Fig. 3—Comparison of CO_2 formation and malic acid disappearance in cucumber juice and in cucumber juice supplemented with 50 mM malic acid fermented with L. plantarum. The initial pH was 5.7 in each experiment. Malic acid disappearance from cucumber juice, --●--; CO_2 formation in cucumber juice, —■—; malic acid disappearance from supplemented cucumber juice, --○--; CO_2 formation in supplemented cucumber juice, —x—.

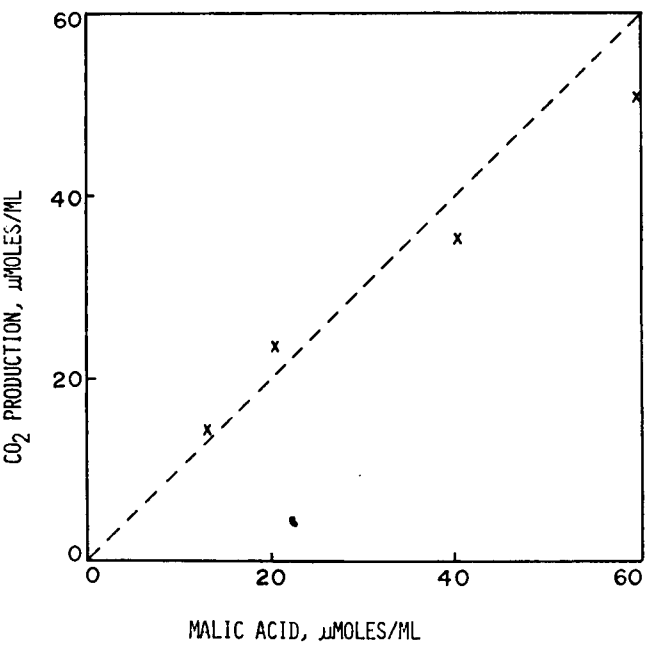


Fig. 5—CO_2 production in cucumber juice supplemented with 6.0% NaCl and malic acid. Determinations were made after incubation at 30°C for 7 days. The dashed line shows the expected CO_2 production if 1 mole of CO_2 were produced per mole of malic acid.

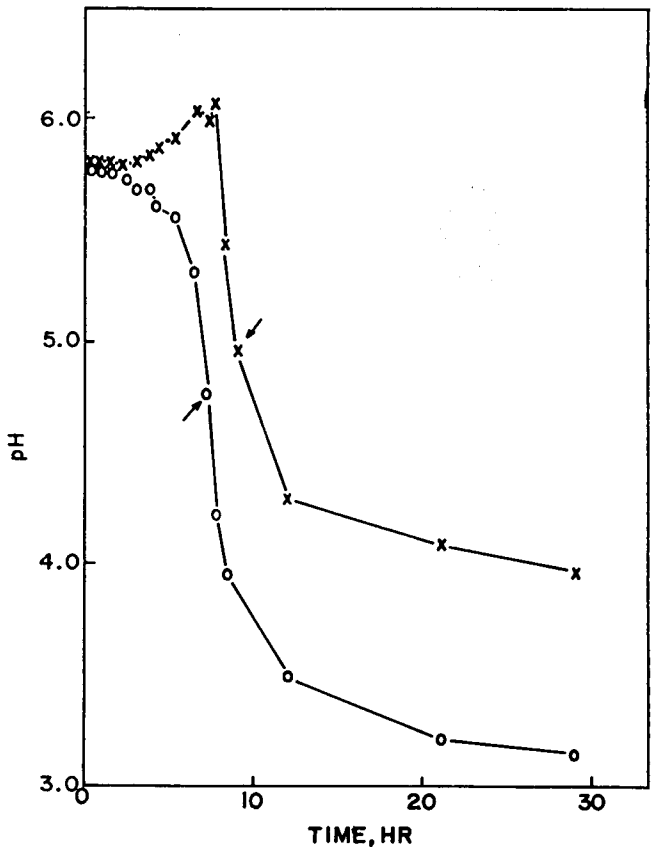


Fig. 4—pH changes during fermentation of cucumber juice by L. plantarum. Arrows indicate the last point at which malic acid was detected. Cucumber juice, ○; cucumber juice supplemented with 50 mM malic acid, x.

be lactate. The uptake of a proton should cause an increase in pH during malate utilization. The lactate will serve to buffer the decrease in pH which will occur as sugars are metabolized to lactic acid.

Fig. 4 shows the pH changes during fermentation in the time course experiments shown in Fig. 3. Arrows indicate the time when malic acid was no longer detected. The unsupplemented cucumber juice shows a slow decline in pH until near the end of the malate degradation period followed by a period of rapid decrease. However, when malate was added to the juice, the expected pH increase was observed. It was followed by a period of rapid pH decline when malate was nearly gone. Also, the pH remained higher with a high initial malate concentration.

The previous experiments were done without addition of NaCl to the cucumber juice. However, commercial cucumber fermentations are normally carried out in 5–6.5% NaCl. Therefore, fermentations were done with cucumber juice to which 6% NaCl and various malic acid concentrations were added. Malic acid was completely metabolized in all fermentations. Fig. 5 shows that in 6% NaCl there was 1 mole of CO_2 formed per mole of malic acid degraded at the lower malic acid concentrations. However, at the higher malic acid levels, the CO_2 formed was less than a 1:1 ratio.

Fig. 6 provides further information on the effect of the malo-lactic reaction on the pH and sugar utilization after 7 days of fermentation. A nearly linear increase in final pH of the fermentation broth was observed as the malic acid concentration was increased. The initial reducing sugar concentration was 103 mM. With the natural level of malic acid, 48.7 mM of sugar remained after fermentation. As the malic acid level of the juice increased, residual sugar decreased until at the highest malic level the reducing sugars

were completely utilized. Etchells et al. (1973) found it necessary to add sodium acetate to commercial fermentations to augment the natural buffer capacity of the cucumbers so complete sugar metabolism would occur. These results are consistent with the proposal of Radler (1966) that malate degradation serves to buffer lactic acid fermentations.

Fleming et al. (1973b) found that in pure culture homolactic cucumber fermentations about half of the CO_2 produced could be attributed to reactions in the cucumber tissue. The other half of the CO_2 was produced during growth of the bacetria. These results have shown that malic acid degradation is the primary source of CO_2 during *L. plantarum* growth in cucumber juice. Based upon the natural level of malic acid in pickling cucumbers (McFeeters et al., 1982), this source could account for the bacteria-produced CO_2 observed by Fleming and coworkers. The source of cucumber-derived CO_2 remains to be determined.

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Ms received 3/24/82; revised 6/4/82; accepted 6/8/82.

Paper no 8233 of the journal series of the North Carolina Agricultural Research Service, Raleigh, NC.

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

The technical assistance of Mrs. Suzanne Armstrong and Ms. Donna Gursky during the course of this work is greatly appreciated.

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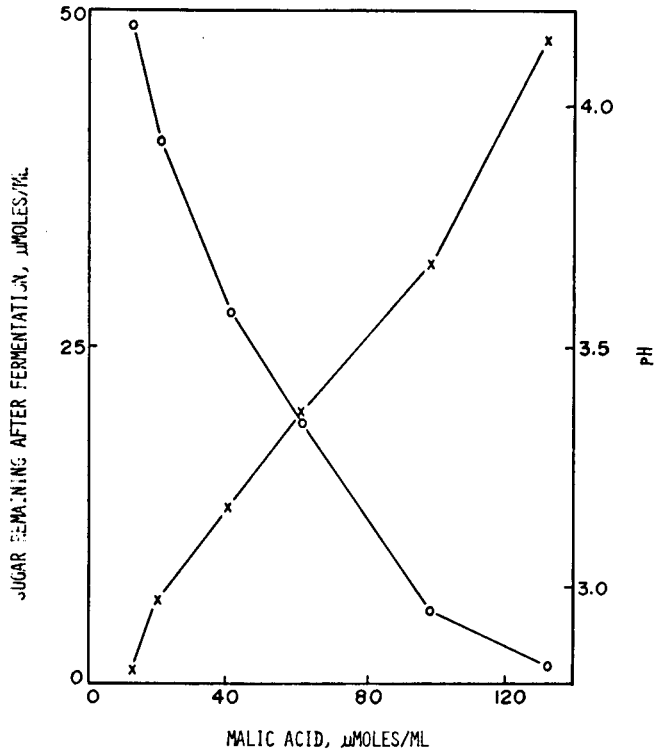

Fig. 6—Final pH and residual reducing sugar in cucumber juice supplemented with 6.0% NaCl and malic acid. Determinations were made after incubation at 30°C for 7 days. pH, x; reducing sugar remaining, o.