AMMONIATED LACTIC ACID FERMENTATION OF FRUIT WASTE SUGARS

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ABSTRACT

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By

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Experiments were conducted to determine the feasibility of converting concentrated pineapple mill waste into a nitrogen-enriched feedstuff for ruminant animals by means of an ammoniated lactic acid fermentation. Commercially canned pineapple juice was diluted to simulate the waste. Homolactic fermentations were conducted with 2-liter batches at constant pH and temperature (45°C) by use of *Lactobacillus delbrueckii* NRRL B-445. The lactic acid produced was continuously neutralized by ammonium hydroxide. The maximal sugar conversion rate was highest (9.9 mg/ml h) with yeast extract (YE) supplementation (10.0 mg/ml) and at pH 6.0, although lactic acid yields were as great (83-85%) at pH 5.5 as at pH 6.0. Contamination was less of a problem at pH 5.5, but sterilization of the medium was still necessary. With the YE supplementation, lactic acid yields increased to over 80% during fermentations at pH 5.0 and greater. As the initial sugar level was increased to 55.0 mg/ml, the maximal sugar conversion rate increased in proportion, but remained essentially constant at higher sugar levels. However, lactic acid yields remained maximal at initial sugar concentrations up to 116.0 mg/ml. Altogether the results indicated that the fermentation might be feasible if conducted at pH 5.5 with a sterilized, supplemented medium containing a high content of fermentable sugar.

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1. INTRODUCTION

Waste utilization in the fruit processing industry has received increased attention due to imposition of governmental pollution limitations. In the Hawaiian pineapple canning industry, for example, although efforts have been made to maximally utilize by-products, a large volume of liquid waste still is channeled into municipal sewers for ocean outfall and subsequent environmental shock (17, 25, 47). Of the 4.4 million tons of pineapple produced in the world in 1973, about 50% was destined for processing (5). In Hawaii alone an estimated 800,000 tons of fresh pineapples were canned in 1971 (30). As a result, in a single Hawaiian plant an average of 527 gallons of waste-water with high biological oxygen demand (BOD) are generated per ton of pineapples processed (12). The total flow of waste per day in a large cannery during processing season can exceed 2.5 million gallons of high-strength effluent, having a BOD of about 27,000 lbs. (F. Aona. Chief, City and County of Honolulu, Department of Public Works, Division of Sewers. 1976. Personal communication.). The sugar level in the composite liquid waste is fairly low (5.0 mg/ml); but two by-products in particular, centrifuge underflow and mill screening fines, are high in carbohydrates and account for most of the BOD. Several million pounds of each of these acidic, semisolid wastes are discarded yearly (61). Should a practical and inexpensive use for these concentrated wastes be found, pollution caused by the composite mill waste could be significantly decreased.

Rather than reducing the BOD of pineapple composite mill waste by standard water-treatment techniques, a bacterial fermentation to produce a ruminant animal feedstuff from the concentrated waste might be exploited. Extensive prior work (43, 67, 74) on the conversion of whey, a waste product from cheesemaking, to a ruminant feedstuff can be used as a model. In the batch process, whey lactose is converted by *Lactobacillus bulgaricus* almost completely to lactic acid, which is continuously neutralized with ammonia (67). The fermentation can be conducted without putrefaction at constant pH (5.5) and temperature (43°C). Of the 55% crude protein in the product, 75-77% is contributed by ammonium lactate, 17% by residual whey proteins, and 6-8% by bacterial cells. The process sequesters ammonia nitrogen in a form that is nontoxic, stable, and nutritious for ruminant consumption. Extensive feeding trials have shown that ammonium salts of lactic, propionic, and butyric acids are equivalent to soybean meal as a nutritious feed ingredient and superior to urea as a commercial nonprotein nitrogen (NPN) supplement (2). Since the cost of chemically synthesized ammonium salts prohibits large-scale use, their alternate production by fermentation of waste substrates has attracted attention.

Because of the increasing demand for animal feeds that are noncompetitive with human food, as well as the pressure to reduce industrial pollution, I undertook to determine the feasibility of converting fruit wastes, as exemplified by pineapple mill waste, into a ruminant feedstuff. The United States pineapple canning industry is concentrated in one area, Hawaii. Because Hawaii has a limited agricultural economy, recycling of waste from the pineapple industry into a feed for the cattle industry has special significance. In developing countries, such as the Philippines, India and Malaysia, competition for food between humans and animals is

more apparent. Recycling of the waste from their expanding pineapple canning industries to a cattle feed would be highly desirable.

To determine the feasibility of this conversion, I examined the applicability of batch fermenting the sugars in pineapple juice into ammonium lactate, at constant pH and temperature. Several lactobacilli were compared for fermentative efficiency, and the need for medium sterilization was considered. The effects of adding yeast extract and of changing pH and initial sugar concentration were studied with respect to maximal sugar conversion rate, lactic acid yield, and residual sugar concentration.

2. HISTORICAL BACKGROUND

2.1 The World Food Problem and Ruminants

With the world population expected to reach over 6 billion by the year 2000, enhanced food production as well as efficient utilization of present resources are essential. In the developing countries, where 86% of the population growth is occurring (80), grain is the staple diet of a majority of the people and can not be diverted for animal feed. This is not so in the U.S. where cattle consumption of grain is not presently competitive with human requirements (21). The situation in the U.S. could change, however, should food shortages develop.

On a world level, the outstanding human food need is for high quality protein to supplement grain diets. About two thirds of the world's people consume food that is deficient in energy, protein and other essential nutrients (76). The world ruminant population, which could make a major contribution in meeting this protein deficit, is far from meager, however. For every 3 people there are 2 ruminants, 60% of which exist in developing countries (21). A partial solution lies in increasing the productivity of the present ruminant population without diminishing food suitable for human consumption.

Compared to other animals ruminants are unique in their ability to consume cellulosic materials that are not efficiently digested by humans. Not only can ruminants scavenge on land unsuitable for crop production, but they also can digest many cellulosic agricultural and industrial by-products that are presently destined for disposal (21). Materials that have been studied with respect to ruminant nutrition include citrus pulp,

cane molasses, newsprint, pineapple bran, and feedlot waste (16, 22, 34, 55, 59, 60, 63, 70).

The unique fermentative capacity of the rumen allows not only for digestion of cellulose wastes, but also for the conversion of nonprotein nitrogen to microbial protein, which is then assimilated by the animal for host-protein needs. A lack of protein is frequently the major factor limiting the use of by-products for feeding (59). Replacing portions of a more expensive protein feed supplement with NPN still allows for adequate ruminant performance (15).

2.2 Ammonium Salts as a Feed Supplement for Ruminants

In 1949 Loosli et al. (51) reported that large quantities of essential amino acids were produced with a ruminant diet containing urea as the sole nitrogen source. Since then, extensive investigations have been conducted on the efficacy of substituting nonprotein nitrogen for protein in ruminant rations. Belasco (8) evaluated an array of nonprotein nitrogen compounds as substitutes for urea. He observed that inorganic and organic ammonium salts, especially ammonium succinate and lactate, exhibited higher rates of nitrogen utilization than urea. Diets with ammonium salts or urea as the only nitrogen source have been sufficient to meet requirements not only for ruminant maintenance metabolism but also for adequate milk production (78), although growth was significantly reduced (62). Subsequent studies have indicated that ammonium salts of acetic, propionic, lactic and butyric acids were superior to urea and comparable to soybean meal as a supplemental nitrogen source in cattle rations (2, 26, 35, 77). The expense of chemically synthesized organic ammonium salts makes large-scale use impractical (35). However, production of these salts by neutralization of waste fermentations with ammonia

provides a practical alternative (26). A successful example of this is the nonaseptic, homolactic fermentation of whey into ammonium lactate (67). The product is superior to urea and equal to soybean meal as a protein supplement (36). The application of this process to other sugary wastes appeared worth consideration, since tentative costs and supplies of protein additives may make the use of alternate NPN feedstuffs essential.

2.3 Fruit Waste Utilization

Most studies on solid and liquid fruit wastes have dealt with treatment rather than utilization. Liquid wastes from the fruit canning industry generally have 10 times stronger BOD values than domestic effluent (4). Over 60% of all cannery wastes are presently being treated in municipal facilities to reduce BOD (49). Rural operations, however, have had to implement their own treatment measures. Methods for reducing BOD have been numerous, including activated sludge treatment (23, 27, 52), anaerobic digestion (9), lagooning (7, 27, 64), spray irrigation (3, 32, 38), and composting (42, 46, 71). Much work has been done on the complete treatment and utilization of the tremendous volume of citrus waste generated annually by the Florida citrus industry. Besides the use of the aforementioned standard techniques (3, 18, 23), citrus by-products such as wet and dried pulp, seed meal, and citrus molasses also have been successfully used in animal rations (11, 16, 45).

Few practical processes for fruit waste utilization have been developed and successfully implemented. Economical utilization of processing wastes depends on lower utilization costs, demand for the recovered product, and minimal investment for research and development (49). Alcohol and yeast production from pear wastes (1, 29, 81) and lactic acid and vinegar production from citrus by-products have been attempted (41,

54, 58). Using a lactobacillus selected from citrus peel juice, Kagan et al. (41) achieved over 90% conversion of sugar to lactic acid. The fermentation lasted 6 days and required high levels of nitrogenous nutrients for maximum yield. Although it was predicted that over 1.5×10^8 lbs. of crude lactic acid could be produced from Florida's annual citrus waste, industrial application apparently has not occurred.

The complete use of all pineapple waste generated has been less extensive than that for citrus wastes. The processing of pineapple for removal of edible flesh uses about 60 to 70% of the whole fruit. Use of the remaining residue varies considerably depending on the area of cultivation. In Southeast Asia and India, reuse has been negligible (40, 72), whereas in Hawaii extensive efforts have been made toward by-product recovery. Methods presently in use in developing countries include vinegar and alcohol fermentation, and silage production (20, 33, 40, 68, 72, 73).

Wastes from large Hawaiian canneries are basically of two types. The solid waste consists of the outer shell and core of the fruit which, after mill juice extraction and syrup recovery (28), are ground and dried for use as a ruminant feed called pineapple bran. Since initial production in 1923, the bran has been a successful livestock feed in Hawaii and has proven economical because of the relatively high cost to import comparable feedstuffs (37). Although the bran is high in total carbohydrates, it is low in crude protein (4.3%) so that supplemental nitrogen must be added for effective use (19, 37). In contrast to Hawaii, the Australian pineapple industry generates wet solid waste which cannot be economically dried to a bran. Instead, the residue, supplemented with protein, is fed in a wet form (48).

The other major waste from pineapple canneries is liquid effluent, amounting to about 15 million gallons per day during peak processing season. About 80% of the waste is cooling water which is directly discharged to nearby streams. The remaining 20% consists of combined waste from trimming, slicing, packing and juice centrifuge flows, and is higher in BOD. This composite pineapple mill waste is channeled to municipal sewers and then pumped to the ocean. Although acute marine pollution was recognized as early as 1944, little work was conducted to alleviate the problem (47). Investigations to date have dealt with treatment by activated sludge as well as anaerobic digestion (12, 47, 53). Treatment by activated sludge was found to be inapplicable on an industrial scale. The anaerobic digestion method reduces the chemical oxygen demand (COD) by 66% only under carefully controlled conditions (53). Centrifuge underflow, the major BOD contributor to the composite mill effluent, has shown promise as a valuable ruminant feedstuff (61). Due to its low protein content, it must be used in conjunction with a protein supplement.

The ammonia-neutralized lactic acid fermentation would not be applicable with pineapple mill effluent due to the low sugar content (5.0 mg/ml) of the waste. However, the fermentation could be used with a more concentrated residue such as the centrifuge underflow or the solid waste before being dried as bran. Both wastes are low in protein, and even a partial conversion of the carbohydrate to ammonium lactate and bacterial cells could significantly elevate the crude protein content. Enhancing the use of centrifuge underflow, which is presently being discarded, would markedly lower the BOD of the composite liquid effluent.

2.4 Lactic Acid Fermentation

Sugar fermentations to produce lactic acid have been utilized since 1881. When substrates containing sucrose or glucose have been used, the homofermentative *Lactobacillus delbrueckii* has been the organism of choice. In lactose fermentations, for example the production of lactic acid from whey, *L. bulgaricus* commonly has been used. Commercial fermentations have required only limited asepsis, because possible contaminants are inhibited by the relatively extreme growth conditions tolerated by the aciduric, thermoduric lactobacilli (14).

Several factors limit the fermentation. The concentration of undissociated acid has been identified as a limiting factor in the lactic acid fermentation with *Streptococcus lactis* (69). A parallel effect was noted during a continuous lactic acid fermentation of whey using *L. bulgaricus*. The undissociated acid that accumulated permitted the fermentation to be free of contamination at a pH higher than that which would normally restrict competing organisms (43). The removal of lactate during growth of *L. delbrueckii* in a dialysis culture system enhanced acid production and maximum cell concentration (31).

Maintenance of a controlled pH has also been shown to increase acid production (50). Not only lactic acid yield and rate of production, but also optimal levels of required growth supplements have been affected by variations in pH (44).

The lactobacilli are also noted for having fastidious nutritional requirements. Vitamins, amino acids and substantial amounts of manganese, potassium, and phosphate ions are frequently necessary for optimum growth and acid production (65, 75).

3. MATERIALS AND METHODS

3.1 Bacterial Culture

Lactobacillus delbrueckii NRRL B-445 (Northern Regional Research Laboratory, Peoria, Ill.) was used throughout the study. This organism was selected as a result of several fermentation trials using *L. delbrueckii* NRRL B-445, *L. lactis* ATCC 4947 and *L. plantarum* strain 3070. *L. delbrueckii* is generally the organism of choice in glucose or sucrose fermentations. Cultures for inoculation were transferred daily into tubes of diluted commercial pineapple juice.

Cell counts were conducted with a Petroff Hausser Bacteria Counter (C. A. Hausser & Son, Philadelphia, Pa.). After appropriate dilution of dense cultures, cells in 15-20 squares were counted and cells/ml was calculated.

3.2 Fermentation Media

Concentrated pineapple mill waste was simulated by the use of canned, unsweetened, Spartan brand Hawaiian pineapple juice (distributed by Spartan Stores, Inc., Grand Rapids, Mich.). The juice, which contained about 120.0 mg sugar/ml, was diluted with distilled water to the appropriate initial sugar concentration called for by each fermentation. The pH was adjusted to the appropriate initial value using ammonium hydroxide (28.8%) and/or hydrochloric acid (6N). Yeast extract (Technical grade; Difco Laboratories, Detroit, Mich.) was added (10.0 mg/ml), as needed, before autoclaving. Lower levels of yeast extract (1.0 mg/ml) have typically been used in lactic acid fermentations. The 10.0 mg/ml concentration

was chosen in this study so that yeast extract would not be limiting with respect to initial sugar levels.

All media used during organism selection consisted of 1:2 diluted pineapple juice at pH 5.5. The media were not autoclaved before inoculation. One medium (Medium A) was not supplemented. Medium B was supplemented with 5.0 g yeast extract/l. Medium C was supplemented with the following (g/l): $MgSO_4 \cdot 7H_2O$ (0.6), $FeSO_4 \cdot 7H_2O$ (0.03), $MnSO_4 \cdot H_2O$ (0.03), KH_2PO_4 (0.5), K_2HPO_4 (0.5), yeast extract (5.0), and trypticase (5.0) (BBL, Cockeysville, Md.).

During organism selection trials, each inoculum consisted of cells harvested from 200 ml of a 12 h culture grown in trypticase soy broth (BBL, Cockeysville, Md.) with 1.0 mg yeast extract and 1.0 mg glucose/ml added.

The medium used for routine culture transfer contained pineapple juice that was diluted 1:2.4 with distilled water, adjusted to pH 6.0 with ammonium hydroxide, and autoclaved.

3.3 Fermentation Equipment and Procedure

Fermentations were conducted with 2 liters of medium in a 5-liter bench-top fermentor equipped with automatic temperature control (Microferm Model MF 105, New Brunswick Scientific Co., New Brunswick, N.J.) and pH control (Model pH-22, and also Model pH-40, New Brunswick Scientific Co., New Brunswick, N.J.).

A 5% inoculum of cells grown 12-15 h at 44°C in unsupplemented 1:2.4 diluted pineapple juice was used. Each batch of medium was autoclaved in the fermentation assembly at 114°C, 12 lb pressure for about 20 min. Other than this initial sterilization, precautions were not taken to maintain asepsis. Before inoculation the fermentation medium

was equilibrated at a temperature of 45°C. A thermometer was used to regularly check the temperature. The unadjusted pH of the pineapple juice was usually 3.7, so ammonium hydroxide was added to elevate the pH to the appropriate initial level. The pH of fermentation samples was periodically checked against a pH meter (Beckman Zeromatic II, Beckman Instruments, Inc., Fullerton, Calif.). A nitrogen blanket was maintained over the medium throughout the fermentation and agitation was constant at 400 rpm.

For each fermentation during organism selection, the pH was maintained at 5.5 and the temperature at 45°C (except for *L. plantarum*, which required 37°C).

With all fermentations, 10 ml samples were removed at regular intervals and stored at -18°C for later analyses. Fermentations were generally terminated after 25-30 h.

3.4 Sample Preparation

For analyses, fermentation samples were first centrifuged for 0.5-1.0 h at 48,000 X g in order to remove bacterial cells and plant material that could interfere with the assays. Analyses (except for total nitrogen) then were conducted with the supernatant fluid.

3.5 Carbohydrate Determination

Total carbohydrate (predominantly sucrose, but some glucose and fructose in the pineapple juice) was determined by the anthrone method of Morris (57). Samples were diluted to contain 50-300 µg equivalents of glucose. To a 1 ml portion in a 25 mm test tube was added 3 ml of distilled water, followed by rapid addition of anthrone reagent and mixing. Anthrone reagent was made by dissolving 2 g of anthrone crystals (The Matheson Co., Inc., Norwood, Ohio) in 1 liter of 95% sulfuric

acid. After standing at room temperature for 15 min, the tubes were assayed in a Spectronic 20 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.) at a wavelength of 540 nm. A standard curve was made by use of a series of tubes containing 100, 200, and 300 μg glucose/ml. A 2 mg glucose/ml solution was diluted 20 times as needed daily for preparation of standards. A blank and a set of standards were included with each determination.

The method of Dubois et al. (24) as modified by Montgomery (56) was used only during preliminary trials to select a lactobacillus. The uncentrifuged fermentation samples were diluted to contain 5-50 μg carbohydrate/ml. A 1 mg sucrose/ml stock solution was diluted to 100 μg/ml and then used to make 3 working standards of 10, 20 and 40 μg/ml. Then 2 ml portions of standards and diluted samples were transferred to a 20 mm test tube, followed by addition of 1 ml phenol reagent and 5 ml concentrated sulfuric acid. Phenol reagent was prepared by addition of 25 g phenol crystals to 500 ml distilled water. After vortexing, the tubes were allowed to stand for 1 h at room temperature. The optical density then was read at 485 nm in a Spectronic 20 spectrophotometer.

Maximum fermentation rates were determined graphically from plots of sugar utilization vs. time.

3.6 Lactic Acid Determination

Lactic acid was determined by two methods. The first analysis was the simplified gas chromatographic procedure of Carlsson (13). A 1 ml sample was applied onto 1 ml of packed cation-exchange resin (Dowex 50W-X8, 100-200 mesh hydrogen form, washed in water; Bio-Rad Laboratories, Richman, Calif.) on glass wool in a Pasteur pipette. After the sample

drained through the resin, the resin was washed twice with 0.5 ml of distilled water. All the fluid from the pipette was collected in a test tube and a 2 µl aliquot was analyzed in a gas chromatograph (Model 810, hydrogen flame detector, F & M Scientific Corp., Avondale, Pa.). The column was 6 ft by 1/4 in O.D. and 2 mm I.D. coiled glass (HP 810, septum to normal FID, Supelco, Inc., Bellefonte, Pa.). The column was conditioned overnight at 250°C and then run isothermally at 220°C. The nitrogen carrier gas was maintained at 10 ml per min, while the hydrogen and air pressures were 7.5 and 11.0 psig, respectively.

The second lactic acid assay was conducted according to the method of Barker and Summerson as modified by Pryce (66). The centrifuged fermentation sample was diluted to contain a final lactic acid concentration of 10-200 µg/ml. A standard solution of 400 µg lithium lactate/ml was diluted to 50, 100 and 200 µg/ml from which fractions were removed for analysis. The remainder of this determination used the following reagents:

Copper reagent - 10.0 g sodium tungstate was dissolved in 800 ml of distilled water. To this was added 22 ml of 85% analytical-reagent grade orthophosphoric acid, followed by 5.0 g copper sulfate ($CuSO_4 \cdot 5H_2O$). The solution then was made to 1 liter with distilled water.

Color reagent - 1.5 g p-hydroxybiphenol (p-phenylphenol) was dissolved in 100 ml dimethylformamide.

Sulfuric acid - analytical-reagent grade.

To 0.2 ml of the sample was added 3.8 ml of copper reagent, followed by mixing. Then 1 ml was transferred to a 25 mm pyrex tube, and 6 ml of sulfuric acid was rapidly added by syringe. The solution was allowed to stand 1-2 min. After 0.1 ml of color reagent was added, the tubes were

mixed and allowed to stand 10 min. Samples and standards were then heated for 90 sec in a steam chamber, cooled in ice, and measured in a Spectronic 20 spectrophotometer at a wavelength of 565 nm.

The lactic acid procedure of Pryce was used routinely for quantification, while the gas chromatographic method of Carlsson was used for product quality determination.

3.7 Total Nitrogen Determination

Total nitrogen was determined by the micro-Kjeldahl procedure (39). Fermentation samples (without centrifugation) were accurately weighed into 10 ml volumetric flasks so that 0-200 ppm nitrogen were present. This was followed by addition of 0.4 g of Pope Kjeldahl salt mixture and then 1 ml of concentrated sulfuric acid, plus a boiling bead. The Kjeldahl salts consisted of a 15.0 g K_2SO_4 - 0.7 g HgO bulk granular mixture (Pope Testing Laboratories, Dallas, Tex.). Samples then were digested on a block digester as the temperature was slowly increased. This was continued until foaming ceased and dense white fumes appeared. Digestion was prolonged for 15 min past clearing of the solution. The flasks were removed and allowed to cool. Samples were then diluted with distilled water to the calibration mark, mixed, and further cooled to near room temperature. The liquid level was again adjusted to the mark, if needed, and samples were poured into screw-cap tubes for storage until analysis with a Technicon AutoAnalyzer II (Technicon Instruments Corp., Tarrytown, N.Y.) after the method of Wall and Gehrke (79).

4. RESULTS

4.1 Typical Fermentation

Figure 1 illustrates the changes in sugar, lactic acid and total nitrogen during a typical lactic acid fermentation of simulated pineapple mill juice by *L. delbrueckii* NRRL B-445. The conversion was homolactic, as evidenced by gas chromatography (Figure 2) and material balance data (Table 1). Figure 2 also indicates that malic acid in the pineapple juice was metabolized, presumably to lactic acid. Continuous neutralization of the lactic acid with ammonia, in combination with protein synthesis contributed to greater than a 4-fold increase in total N per unit wet weight of juice. The increase in total nitrogen is actually greater than this, because it was necessary to raise the pH of the juice from 3.7 to 6.0 with ammonium hydroxide before the fermentation could commence. The adjustment of three factors, pH, presence of yeast extract, and initial sugar concentration, affected the rate of carbohydrate fermentation, and the yield of lactic acid.

4.2 Organism Selection

L. delbrueckii was selected because of fewer contamination problems. The two thermoduric lactobacilli, *L. delbrueckii* and *L. lactis*, fermented the enriched media to about equivalent residual levels (Figures 3 and 4). In those fermentations exhibiting contamination, the desired lactobacillus constituted about 80% of the cell population and lactic acid was still the predominant product as evaluated by gas chromatography. The two thermoduric lactobacilli exhibited about equivalent fermentation times, 15 h and 21 h, to reduce the sugar levels in Media C and B, respectively, to

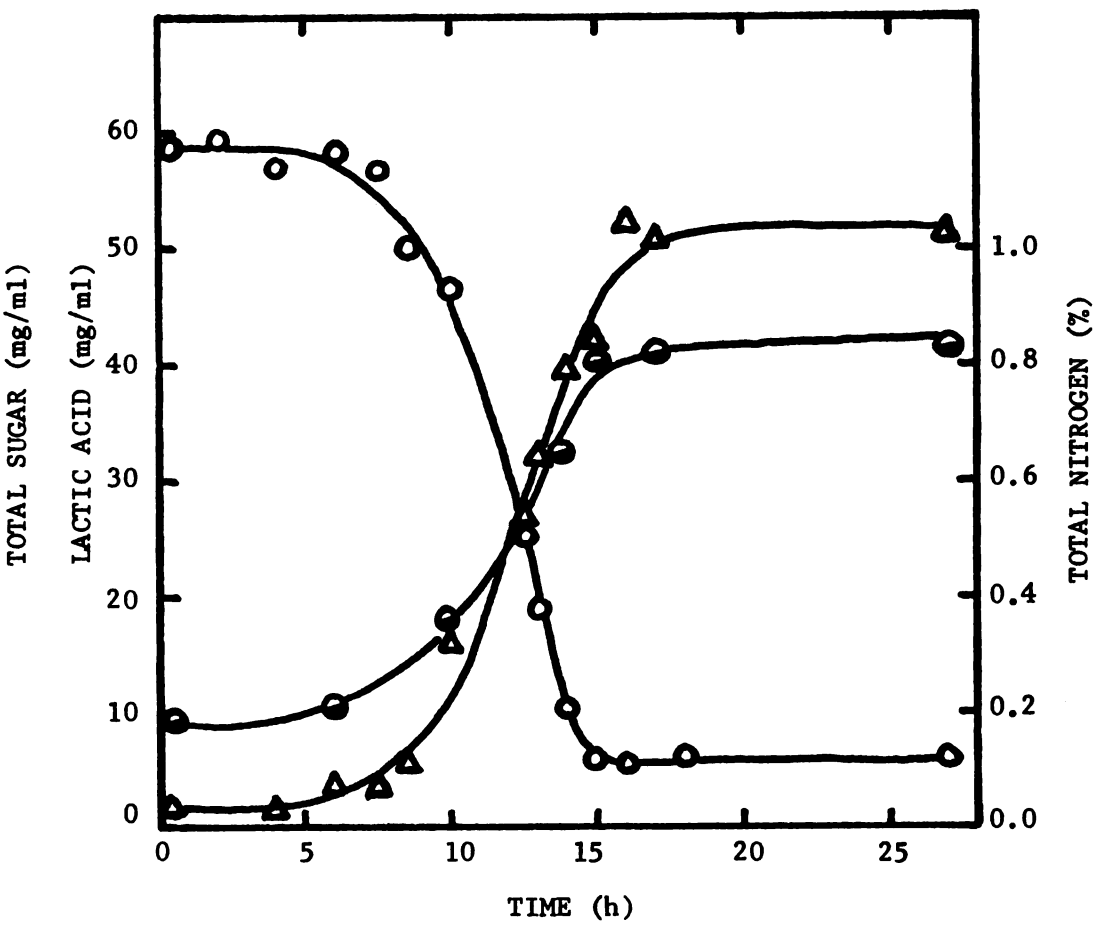

Fig. 1. Change in total carbohydrate (○), lactic acid (△), and total nitrogen (◒) levels during fermentation by L. delbrueckii of pineapple juice at pH 6.0 and with yeast extract added (10.0 mg/ml).

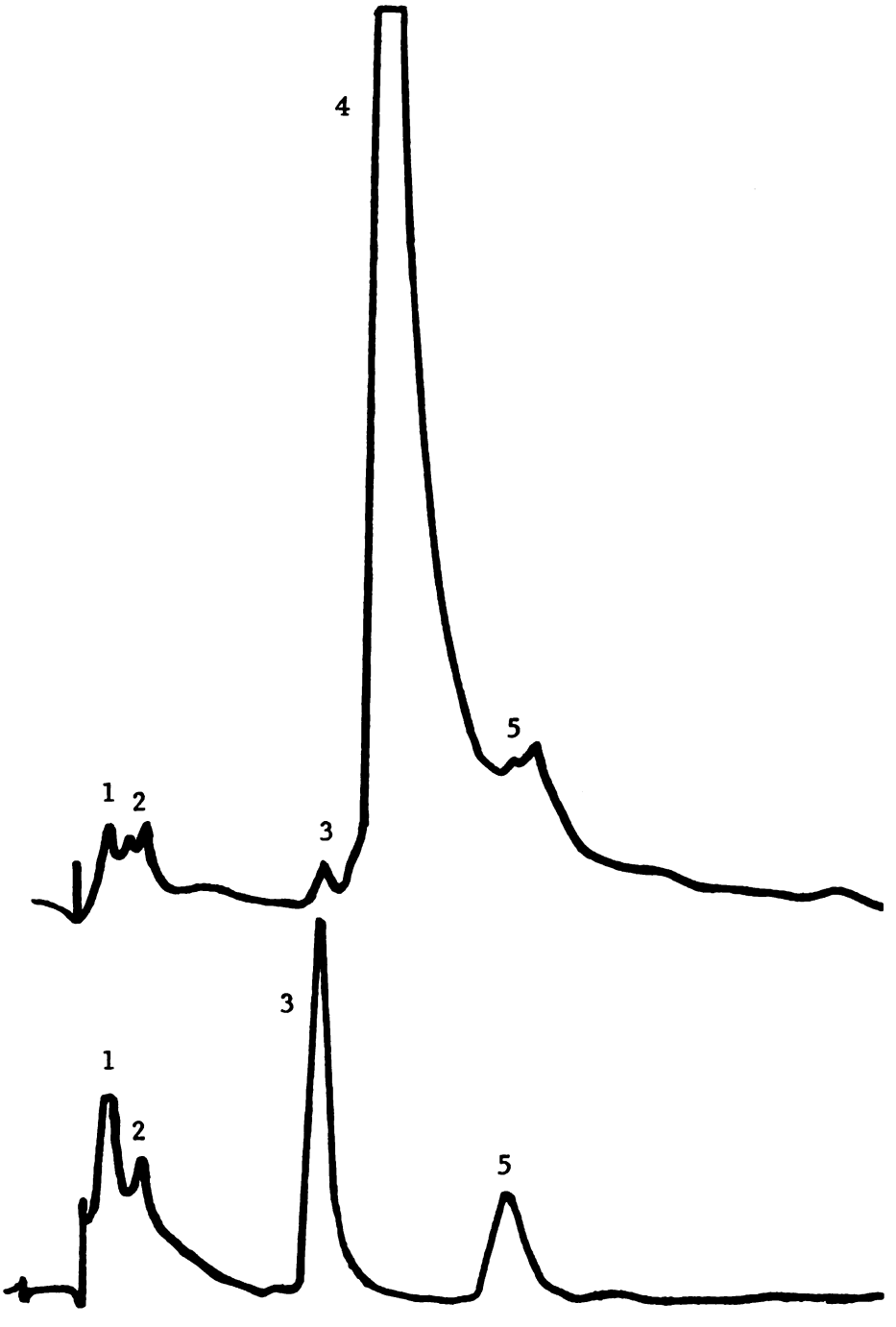

Fig. 2. Gas chromatogram of initial substrate (bottom) and final product (top) from a fermentation conducted according to procedures as described in Materials and Methods; attenuations = 16 and 32, respectively. Symbols: 1, background; 2, unknowns; 3, malic acid; 4, lactic acid; 5, citric acid.

Table 1. Material balance data for a fermentation of simulated pineapple mill waste supplemented with yeast extract.

Fermentation[a] time (h)	A Sugar (mg/ml)	B Lactic acid (mg/ml)	A+B Sum (mg/ml)[b]
0.0	59.2	0.0[c]	59.2
3.5	57.6	0.0	57.6
5.5	53.1	1.6	54.7
7.0	56.4	2.3	58.7
10.0	48.8	9.6	58.4
12.0	36.5	18.3	54.8
13.0	30.8	23.8	54.6
14.0	23.9	30.1	54.0
15.0	21.2	35.4	56.6
16.0	13.1	40.6	53.7
17.0	9.1	42.6	51.7
18.0	6.4	50.3	56.7
28.0	6.4	50.3	56.7

[a]Fermentations conducted at pH 6.0 in a medium containing 60 mg sugar and 10 mg yeast extract/ml.

[b]Mean = 56.0; standard deviation = 2.2.

[c]The gas chromatogram (Fig. 2) of a 0.0 h fermentation sample indicates the absence of lactic acid, while the Pryce lactic acid assay indicates 3.3 mg/ml, which is due to high sugar concentrations causing false lactic acid readings. Therefore, 3.3 mg/ml was subtracted from the 0.0, 3.5, 5.5, and 7.0 h values for lactic acid.

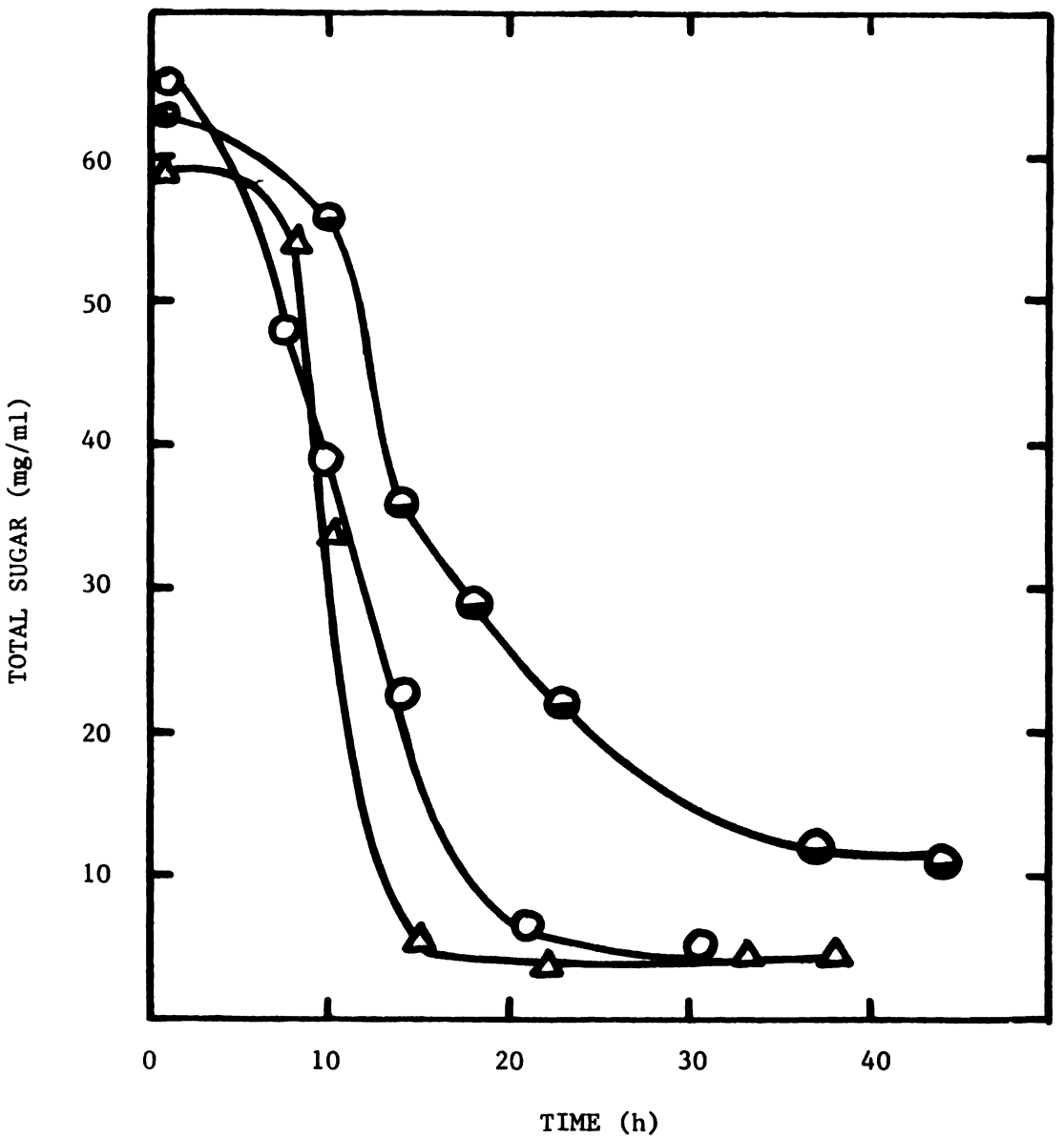

Fig. 3. Sugar utilization by L. delbrueckii NRRL B-445 in Media A (◒), B (○), and C (△) at pH 5.5(refer to conditions as described in Materials and Methods).

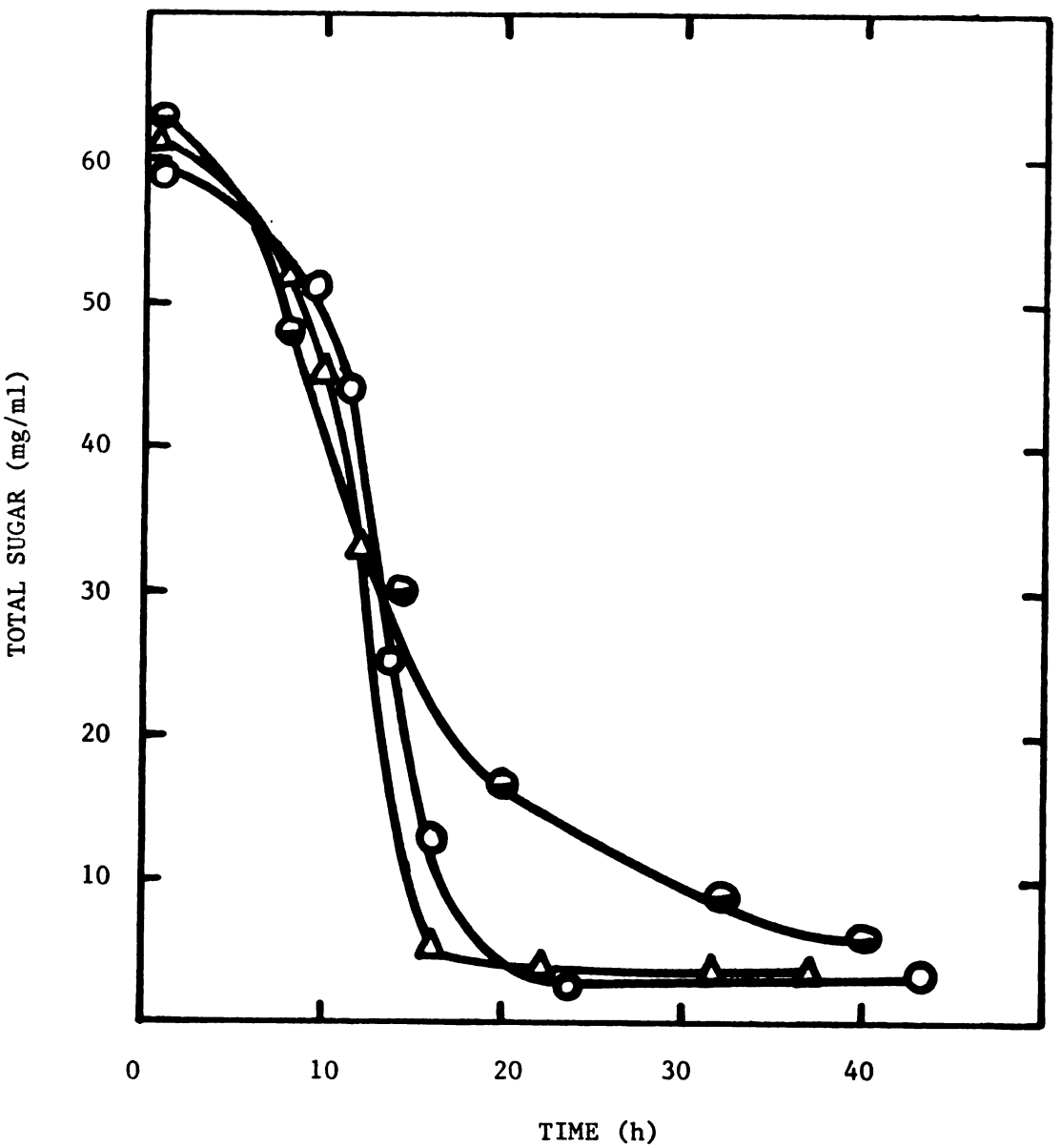

Fig. 4. Sugar utilization by L. lactis ATCC 4947 in Media A (●), B (○), and C (△) at pH 5.5 (refer to conditions as described in Materials and Methods).

5.0–6.0 mg/ml. *L. plantarum* was eliminated as a possible organism because not only did it require a longer fermentation time than the other two lactobacilli to reach a sugar concentration of 5.0–6.0 mg/ml in Medium B (Figure 5), but it also had an optimum temperature of 37°C, which was less restrictive for contaminants than was 45°C.

4.3 Effect of pH

A change in pH within the range of 4.5–7.5 had a pronounced effect on the maximum rate of fermentation (Figure 6). A maximum fermentation rate of 9.9 mg/ml h occurred at approximately pH 6.0 in supplemented media; however, the yield of lactic acid was fairly constant at pH values of 5.0 and above (Table 2). Extreme pH values of either 4.5 or 7.5 effectively depressed the rate of carbohydrate utilization.

The influence of pH on the yield of lactic acid was greatest in unsupplemented pineapple juice. As indicated in Table 2, an increase in pH from 4.5 to 5.5 with this medium resulted in over a 100% increase in lactic acid. In a medium supplemented with yeast extract a similar pH adjustment caused only a 41% increase. The yield of lactic acid, however, was much higher in the supplemented medium as compared to the unsupplemented one. In both media the highest yield occurred at pH 5.5.

4.4 Effect of Yeast Extract Addition

Sugar utilization was significantly improved by the addition of yeast extract (Figure 7). Without yeast extract, fermentations were incomplete after 25–30 h. However, upon addition of yeast extract, residual sugar levels below 10.0 mg/ml were obtained.

In Figure 8 is illustrated the course of a fermentation with and without a nitrogenous supplement. There was over a 5-fold increase in

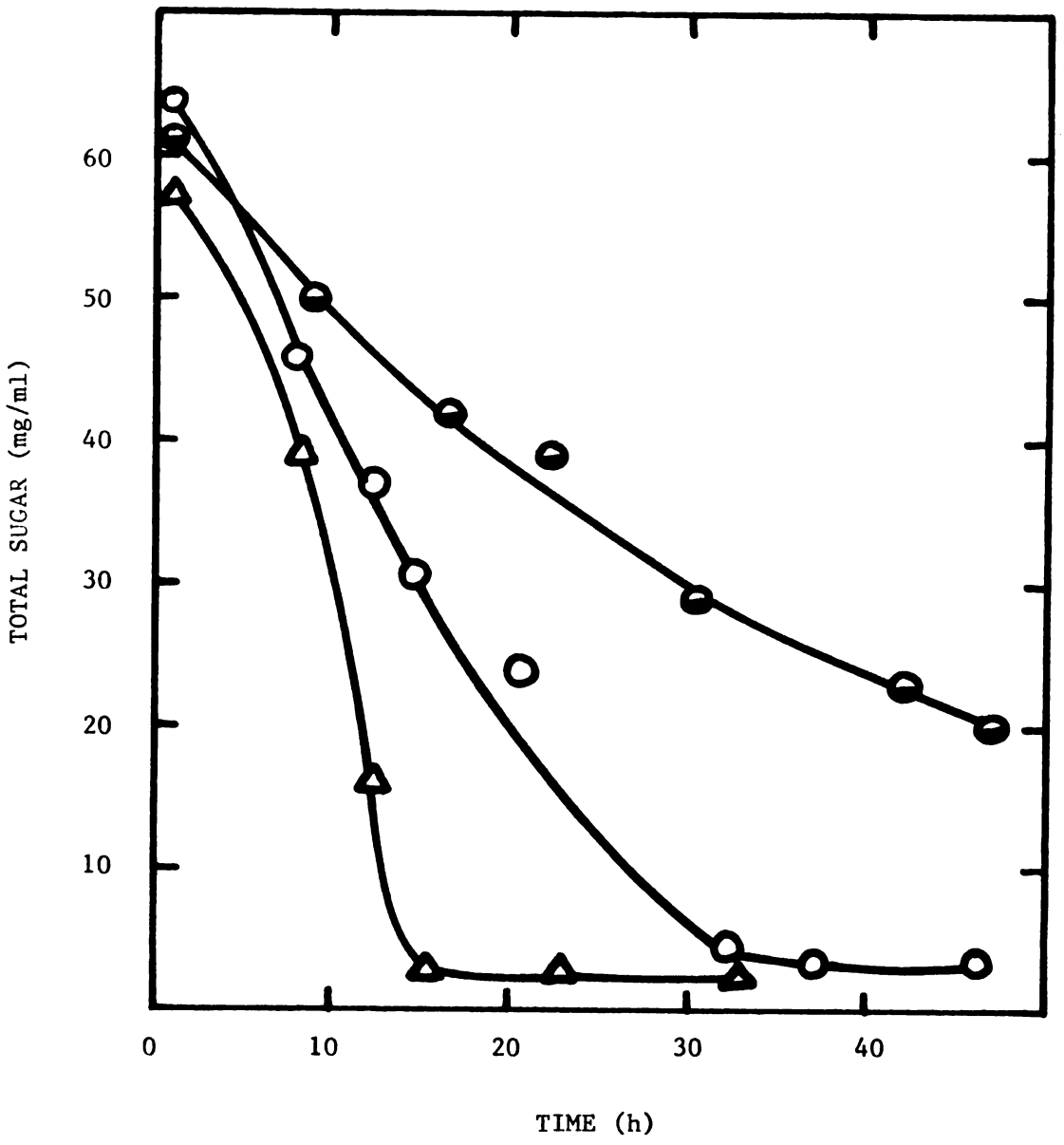

Fig. 5. Sugar utilization by L. plantarum strain 3070 in Media A (●), B (○), and C (△) at pH 5.5 (refer to conditions as described in Materials and Methods).

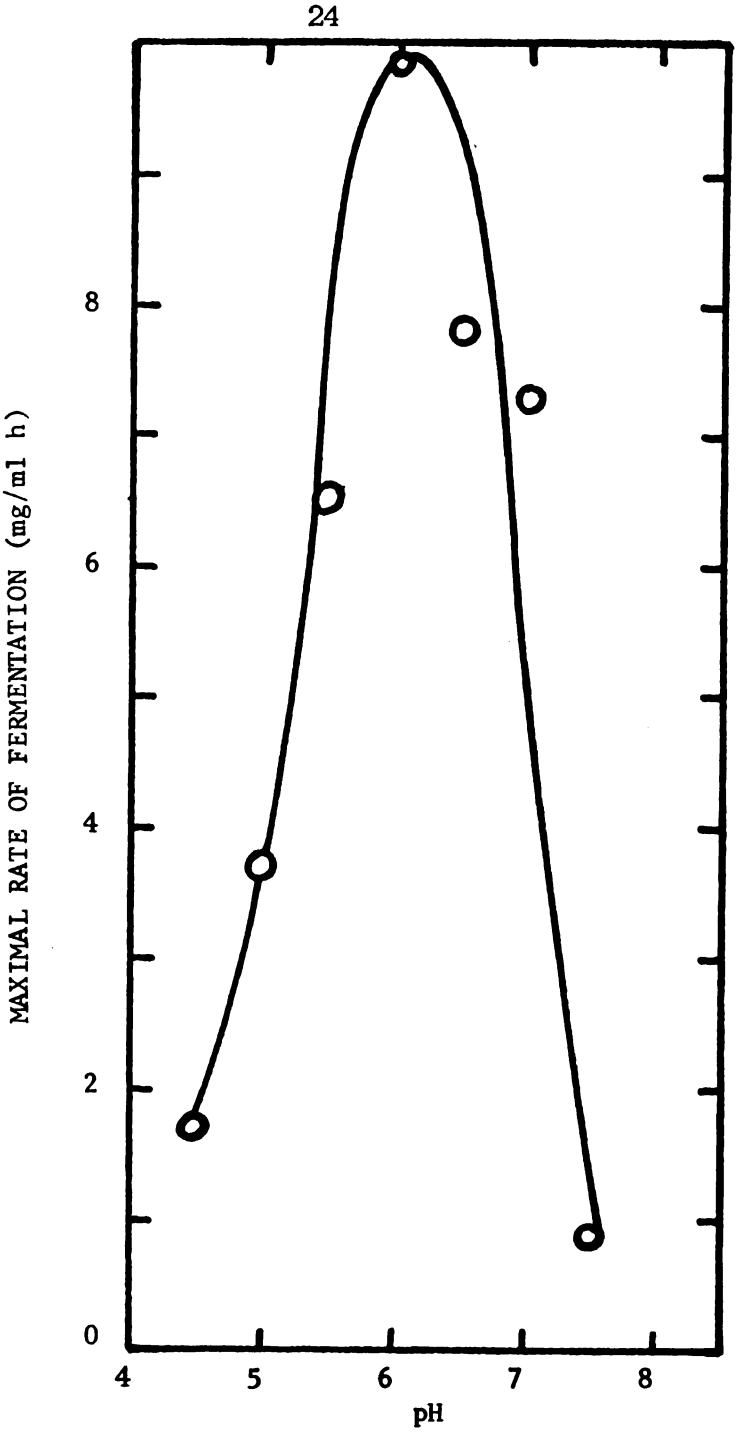

Fig. 6. Effect of pH on the maximal rate of sugar fermentation by L. delbrueckii. The fermentation medium contained 10.0 mg yeast extract and 55.0 - 65.0 mg initial sugar/ml.

Table 2. Effect of pH and yeast extract on the yield of lactic acid during fermentation of pineapple juice by *L. delbrueckii*.

pH	Lactic acid yield (%)		Crude protein (%)[b]
	With yeast extract[a]	Without yeast extract	
4.5	60.0	21.6	3.5
5.0	83.5	30.3	4.6
5.5	85.0	45.4	4.8
6.0	83.7	33.5	5.1
6.5	82.1	31.0	4.7
7.0	80.4	--	5.4

[a]Medium contained about 60.0 mg initial sugar and 10.0 mg yeast extract/ml.

[b]Calculated after completion of the yeast extract supplemented fermentation.

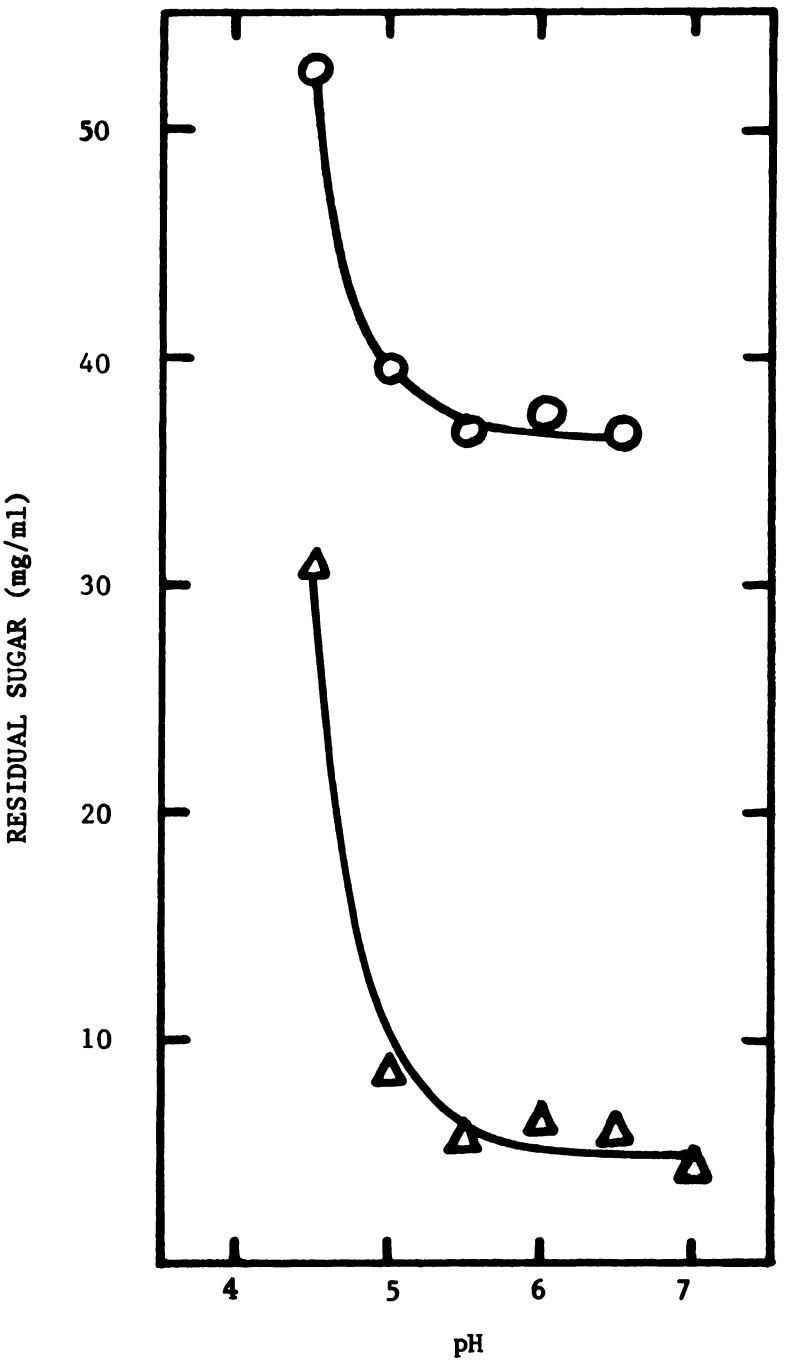

Fig. 7. Effect of yeast extract addition on utilization of pineapple juice sugars by L. delbrueckii. Residual sugar levels after 25 - 30 h of fermentation were evaluated in media with 10.0 mg yeast extract/ml (△) and without yeast extract (○). The initial sugar level was about 60.0 mg/ml.

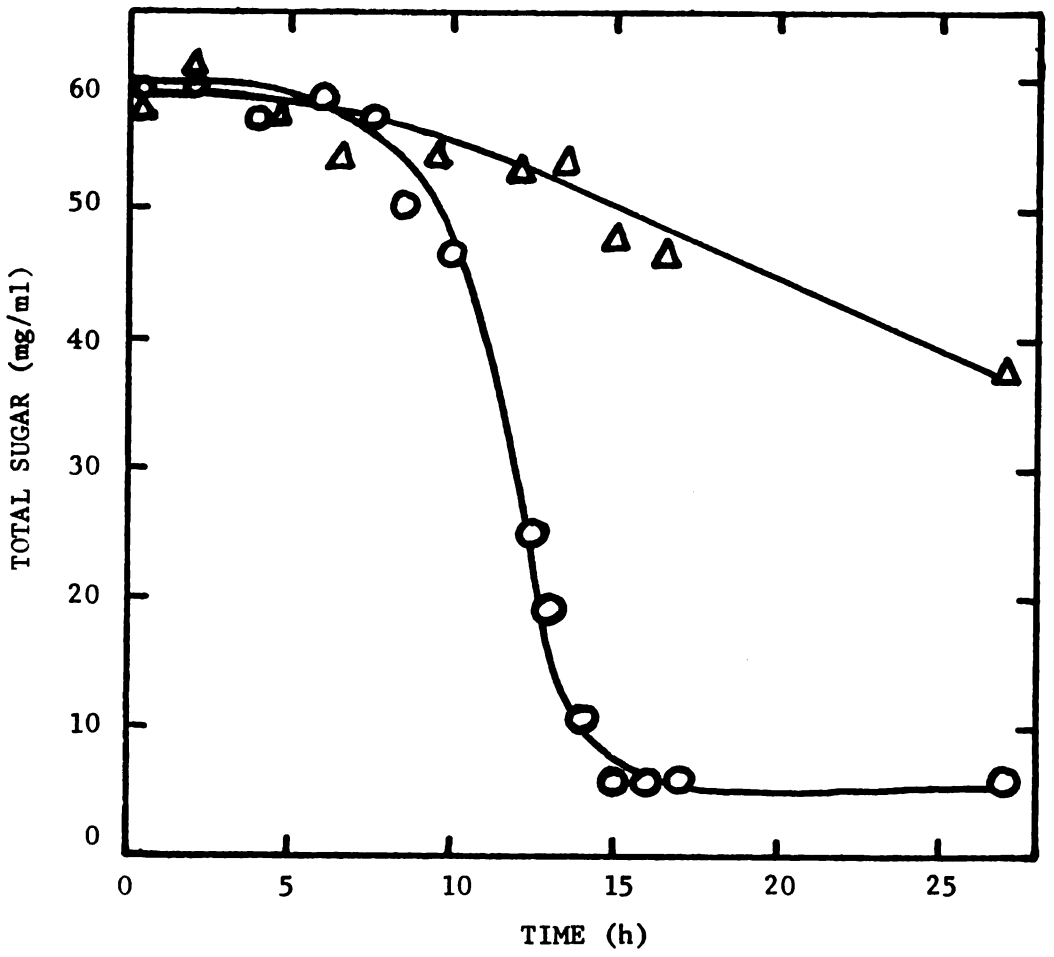

Fig. 8. Effect of yeast extract addition on the course of a typical fermentation of pineapple juice at a constant pH of 6.0. Symbols: (○) 10.0 mg yeast extract/ml, (△) no yeast extract added.

the rate of fermentation in supplemented pineapple juice as compared to the unsupplemented medium. This of course caused a significant reduction in fermentation time in the enriched medium to 16 h.

A comparison of Figures 3 and 8 indicated that *L. delbrueckii* more efficiently utilized unsupplemented pineapple juice during organism selection trials than in later fermentations. The difference in inocula and operating pH between the two fermentations would not be expected to cause such a change in sugar utilization. The reason for the discrepancy was not known and was not investigated further.

4.5 Effect of Initial Sugar Concentration

Between initial sugar levels of 5.0-55.0 mg/ml, the maximum rate of fermentation increased in proportion to increases in sugar concentration (Figure 9). Above initial sugar levels of 60.0 mg/ml at pH 6.5, the maximum rate of utilization was less dependent on sugar concentration. Fermentations conducted in media with 77.0 to 116.0 mg sugar/ml plus yeast extract (Table 3) still produced final carbohydrate concentrations of 5.0-10.0 mg/ml and lactic acid yields of approximately 82-85%. The fermentation time was extended to only 19-24 h. Throughout the studies with yeast extract supplemented media, residual sugar levels of 5.0-10.0 mg/ml were usually obtained.

4.6 Culture Purity

The initial objective of this study was to accomplish the pineapple waste conversion in a completely nonaseptic manner. However, repeated problems with contaminants were encountered. Although contamination was less of a problem at pH 5.5 than at higher pH values, sterilization of all media was still necessary.

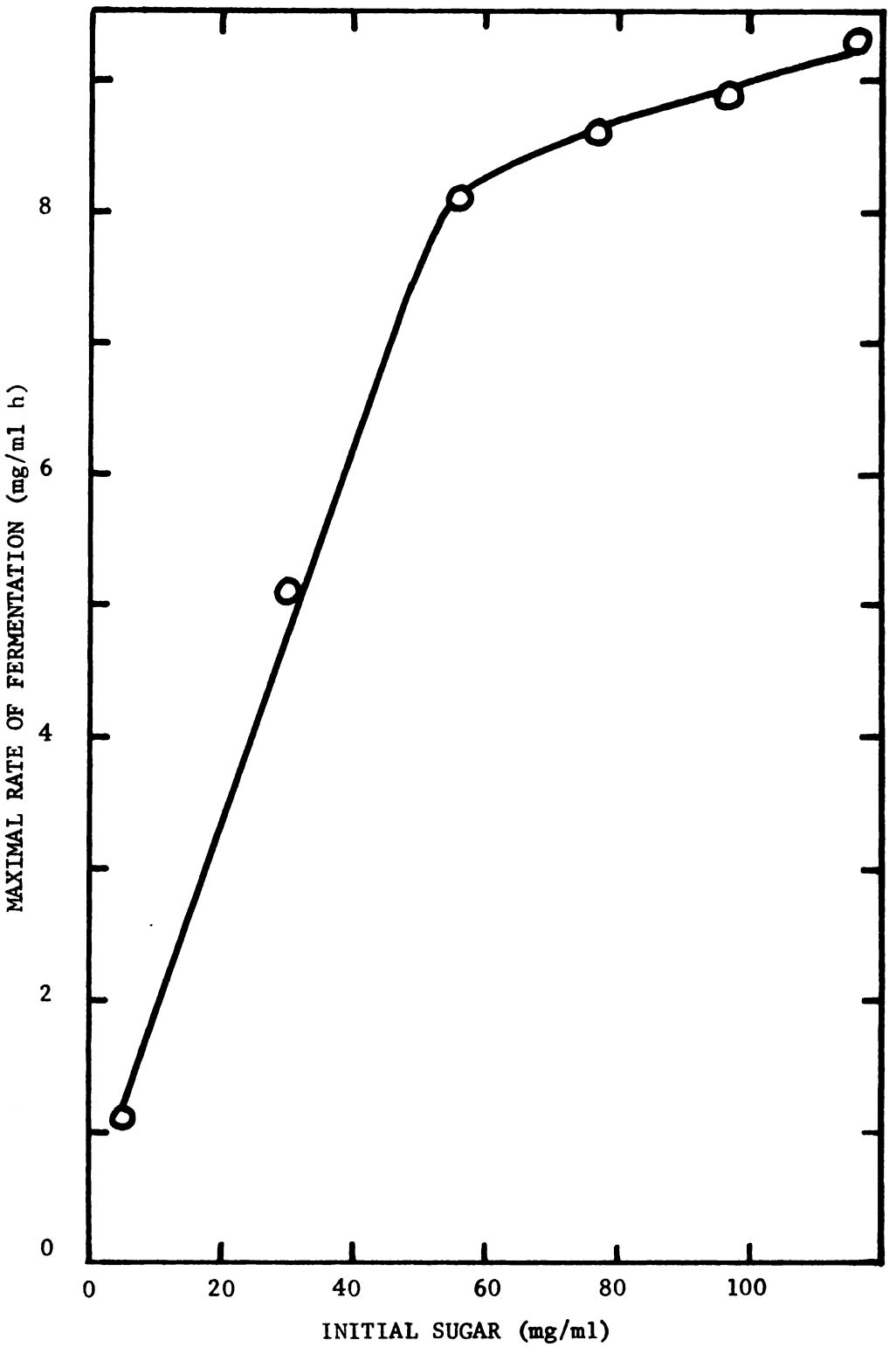

Fig. 9. Effect of initial sugar level on maximal rate of sugar fermentation by L. delbrueckii. Fermentations were conducted at a constant pH of 6.5 and supplemented with yeast extract (10.0 mg/ml).

Table 3. Lactic acid yield and sugar utilization at various initial sugar concentrations during fermentation of pineapple juice by *L. delbrueckii*.

Initial sugar (mg/ml)	Fermentation[a] time (h)	Residual sugar (mg/ml)	Lactic acid yield (%)
31.0	18	5.0	77.4
56.0	17	5.0	84.9
77.0	19	6.0	83.8
97.0	20	6.0	82.9
116.0	24	9.6	81.9

[a]Fermentations were conducted at pH 6.5; 10.0 mg yeast extract/ml was added.

5. DISCUSSION

Application of the ammoniated lactic acid fermentation for the processing of fruit wastes requires that the fermentation is operated in an efficient and inexpensive manner and that the product is useful as a ruminant feedstuff. In the present study the lactic acid yield, the rate of fermentation and the residual sugar remained almost constant in supplemented media containing 55.0-116.0 mg initial sugar/ml. This indicates that higher concentrations of sugar might be efficiently fermented. In contrast, initial lactose levels of up to only 70.0 mg/ml can be fermented efficiently by *L. bulgaricus* grown at pH 5.5 in whey (67). With an initial concentration of 115.0 mg lactose/ml in whey, there remained 40.0 mg/ml after 16-24 h, indicating incomplete fermentation. Because *L. delbrueckii* can utilize high levels of initial sugar, the product from the fermentation of pineapple waste might be attractive as a ruminant feedstuff if a material high in fermentable carbohydrate is used as a substrate. The product, due to its higher content of ammonia nitrogen, would be more suitable for use as an NPN feedstuff, and would require less concentration to reach a nitrogen level comparable to that in other crude protein supplements. Liquid effluent from pineapple canning plants is low in carbohydrate (less than 10.0 mg/ml), which would not make a lactic fermentation worthwhile. However, if either semisolid centrifuge underflow waste or ground pineapple shells

and cores could be partially fermented with ammonia neutralization, a more nutritious nitrogen enriched feedstuff would be produced.

The lack of certain nutrients required for the growth of *L. delbrueckii* in simulated pineapple mill waste significantly depressed lactic acid production. An additive rich in growth factors such as yeast extract was required for efficient lactic acid production. The maximum yield of lactic acid in yeast extract supplemented and in unsupplemented pineapple juice was 85% and 45%, respectively, at pH 5.5. In comparison, the lactic fermentation of yeast extract enriched wheat grit mash with the same strain of *L. delbrueckii* gave a lactic acid yield of 80% occurring at pH 4.6 (44). The optimum yield in unsupplemented mash was 70% at pH 6.0. Supplementation permitted satisfactory yields of lactic acid at low pH, which was not true in the present investigation, where optimum yields occurred at pH 5.5 regardless of nutrient addition. Locating a readily available and less expensive growth supplement than yeast extract is a limiting factor in practical application of this fermentation process. During fermentation of ground pineapple shells or cull fruit, some, but not all, of these nutrient requirements might be supplied by components in the skin.

Limiting factors in the use of fermented pineapple waste as a ruminant feedstuff would be the cost of ammonia and the cost of initial sterilization. Drying the product would significantly increase the crude protein content but would also increase the cost. For the simulated pineapple waste (60.0 mg sugar/ml) fermented at pH 5.5, the final crude protein level was 4.8%. A 10-fold concentration would yield a

material with over 40% crude protein. This is an appreciable amount, since the concentrated product from the ammoniated lactic acid fermentation of whey has 55% crude protein (67).

In summary, the process of crude protein enrichment of pineapple mill waste by an ammoniated lactic acid fermentation might have potential use if several conditions are met. The fermentation should be conducted at constant low pH (5.5) and high temperature (45^{o}C). Although the maximal rate of sugar utilization by *L. delbrueckii* in supplemented pineapple juice occurred at pH 6.0, the fermentation should be conducted at pH 5.5, since the lactic acid yield is slightly higher and contamination is less of a problem at the lower pH. Initial sterilization may be necessary, however. A waste with a high content of carbohydrate and supplemented with a cheap source of organic nitrogen growth factors should be used. Concentrating the product, if economically allowable, would significantly enhance the total nitrogen level.

In developing countries, such as India or the Philippines, where growth of the food processing industry coincides with depletion of cattle feeds (6, 10), the procedure holds promise as a means of both reducing environmental pollution and producing a protein enriched ruminant feed supplement. This supplement in conjunction with a suitable energy feed could partially substitute for more expensive protein additives, and could also improve animal productivity in areas where protein supplementation is presently inadequate.

In Hawaii, with an economy limited mainly to pineapple and sugar cane production, the recycled pineapple waste could reduce the dependence of the growing cattle industry on expensive imported protein supplements.

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