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ABSTRACT

CONTINUOUS LACTIC ACID FERMENTATION OF WHEY TO PRODUCE A FEED SUPPLEMENT HIGH IN CRUDE PROTEIN

by

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Continuous lactic acid fermentation was shown to be a simple and efficient process for converting whey into a source of crude protein (N x 6.25) for ruminants. Lactic acid bacteria were used to convert whey lactose to lactic acid, which in turn was neutralized with anhydrous ammonia. The process was operated non-aseptically in a 14-liter fermentor for 42 days without degeneration of the product and with an actual increase in efficiency of conversion.

The effects on the continuous fermentation on retention time, number of fermentor stages and pH were investigated. An increase in retention time up to 15 hr resulted in an increase in lactose conversion, but only marginal improvement was realized by further increasing the retention time. With a retention time of 15 hr and a pH of 5.5 in a single-stage fermentor, the residual lactose concentration was 0.7%. Increasing the retention time to 31 hr resulted in a residual lactose concentration of 0.6%. However, by employing two fermentors in series with a total retention time of 31 hr, it was possible to reduce the residual lactose to less than 0.1%. Increasing the pH from 5.5 to 5.8 resulted in a substantial reduction in the residual lactose concentration, but further increasing the pH to 6.0 resulted in only a small additional reduction.

A published mathematical model for lactic acid fermentation was modified and used to simulate the whey fermentation. A close fit was obtained between the simulated and the experimental results. Use of the model indicated that lactic acid is produced not only as a function of bacterial growth but also of maintenance metabolism. The simulation predicted that a three-stage lactic acid fermentation would give little improvement over a two-stage process.

After 14 days of the continuous fermentation, a significant increase began to take place in the ability of the culture to ferment lactose to lactic acid. After 19 days, the increased fermentation rate became stabilized. The change in the culture made it possible to increase the throughput rate by a factor of 3 and yet retain the same degree of lactose conversion.

The predominant fermentation product was shown to be lactic acid. Gas chromatography was used to detect other metabolic products, but only traces ($<0.2\%$) of ethanol and acetic acid were observed. The fermented product contained approximately 9 times as much crude protein as the unfermented whey.

Batch fermentations were used to demonstrate that sources of growth factors can be used to reduce the fermentation time. The addition of yeast extract (0.20%) or cornsteep liquor (0.25%) reduced the fermentation time to one-half of that experienced with unsupplemented whey.

Batch fermentations were also used to demonstrate that product inhibition rather than substrate exhaustion accounts for the progressive decline in fermentation rate observed in batch lactic acid fermentations. Both ammonium lactate and calcium lactate inhibited the lactic acid fermentation, but the former was about twice as inhibitory as the latter. The interaction of pH and product concentration was evaluated utilizing a model relating pH to the biological activities of weak acids and bases.

CONTINUOUS LACTIC ACID FERMENTATION OF WHEY
TO PRODUCE A FEED SUPPLEMENT HIGH
IN CRUDE PROTEIN

By

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To Carole for her love, patience and
understanding throughout this investigation.

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3. INTRODUCTION

The American dairy industry annually discards 22 billion pounds of whey on fields or in municipal sewers or markets the whey as products which return marginal or negative profits (2, 71). About half of the milk solids remains in the whey after separation from the cheese curd. With cheese production increasing and with greater public awareness of environmental pollution, cheesemakers are under increased pressure to find alternative means of salvaging this enormous supply of nutritious milk solids.

The magnitude of the whey problem is not as great in Michigan as elsewhere, since Michigan produces only 3% of the nation's cheese. However, for the individual cheese producer within the State, the problem is acute. Pressure is also upon processing plants for potatoes, sugar beets, cherries, vegetable products and other carbohydrate foods to find economical means of managing their wastes. A process for coping with whey is potentially applicable to these products as well.

A potential solution exists in the fermentative conversion of whey into a feed supplement containing a high concentration of crude protein ($N \times 6.25$). There is a broad base of fundamental technology and several specific processes for such a fermentation (see the historical section below). These processes use lactic acid bacteria to convert the whey lactose into lactic acid, which in turn is neutralized with ammonia. The fermented product contains approximately 8 times more crude protein than whey. After condensation, the product contains about 50% crude protein and serves about as well as soybean meal in the rations of ruminants.

The above process has not been developed and commercialized in the past due in part to the relatively low price of alternate protein supplements, notably soybean meal, and to the limited information on the performance of ammonium lactate in the rations of ruminants. In the past year, however, dramatic changes took place in the price of all protein supplements. The commodities market, reflecting the world food shortage and particularly the protein shortage, reacted with higher prices for all protein supplements. It is now economically feasible to consider alternate sources of crude protein, such as ammoniated whey, which in the past were uncompetitive. In addition, the safety and efficacy of ammoniated whey as a source of crude protein is now demonstrated.

The problem of whey disposal and the improved market for protein supplements served as the impetus for research to improve the process for fermenting whey to produce a source of crude protein for cattle. The primary objective was to determine the feasibility of a continuous fermentation process, which potentially is much more efficient than a batch process. During the study, the following factors of continuous fermentation were evaluated: retention time in fermentor, number of fermentor stages, pH of fermentation, culture stability under non-aseptic conditions, and product quality.

In addition to the laboratory experimentation, a mathematical model of the continuous fermentation was developed and used to simulate the process on a digital computer. In ancillary studies with batch fermentation, stimulation was demonstrated by the addition of crude sources of growth factors; and inhibition was demonstrated by the addition of the product, ammonium lactate.

4. HISTORICAL REVIEW

4.1 *Feed Supplement from Whey*

Whey is the liquid fraction that results when fat and casein are removed from whole milk in the process of cheesemaking. Whey contains roughly half of the solids and most of the vitamins and minerals of the whole milk from which it is derived (67, 68). Ten pounds of milk yield about nine pounds of whey and one pound of cheese. Whey contains about 93.1% water, 0.3% fat, 4.9% lactose, 0.9% protein, 0.6% ash and 0.2% lactic acid or about 7% total solids (41).

Of the 1.40 billion pounds of whey solids available in the U.S., approximately 700 million pounds are processed and used as by-products. About 250 million pounds of solids are marketed as edible products for human consumption (70), while the balance (approximately 450 million pounds) is marketed as animal feed (67, 68). The edible products are used in baked goods, ice cream, sherbet, cake mixes, batter mixes and the like (68), whereas milk replacers for calves and pigs are the biggest markets for feed-grade whey (67). The lack of suitable markets limits the quantity of whey sold for human consumption, and the return to the whey processor from feed-grade whey is barely enough to cover the cost of processing. The animal industry could use all of the whey solids produced in the U.S., but only if whey prices were below production costs (68). Pricing below costs is an unsound business practice which few whey processors can afford to follow. Consequently, alternative methods must be developed for utilizing the 1.15 billion pounds of whey solids which are an economic burden to the cheese manufacturer and an ecological burden for the public.

As early as 1945 it was recognized that whey and similar materials could be converted to sources of crude protein for ruminant animals. Two Dutch patents (27, 50) were issued for batch fermentation processes in which the traditional method of converting sugar-bearing products to lactic acid was followed, with the exception that ammonia was used as the neutralizing agent. A culture of a homofermentative lactic acid bacterium, such as *Lactobacillus bulgaricus*, was used to convert the whey lactose to lactic acid. During the fermentation, the lactic acid produced was neutralized intermittently with ammonia so that the pH in the fermentor remained between 5.0 and 5.8. The fermented product, condensed to 10% of its original volume, was reported to be a good protein supplement for ruminants.

In 1958 a U.S. patent was granted for essentially an identical fermentation process (10). In the same year a more detailed description of a method for manufacturing a high-nitrogen, low-lactose product from whey was published in the scientific literature by Arnott, *et al.* (3). This process was virtually the same as the two Dutch and the American patents. An important observation was that the fermentation time could be considerably reduced by the use of automatic pH control. About 80% of the crude protein in the product, referred to as "ammoniated whey", was in the form of inorganic ammonium ions and the remaining 20% was true protein in the form of whey proteins and bacterial cells.

Feeding trials were conducted utilizing "ammoniated whey" as a protein supplement for cattle (21). For most of the cattle, feed consumption was reduced when the supplement was included in the concentrate portion of the ration. However, those cattle which maintained high consumption rates performed very well. The conclusion of the study was that there may be a palatability problem with the supplement; however, the

supplement that was consumed performed very well. In a subsequent study, in which "ammoniated whey" was included in the total ration rather than in the concentrate portion of the ration, no palatability problem was found with the supplement. In fact, the ammoniated whey appeared to perform as well as soybean meal when used as the sole source of supplemental nitrogen (45).

"Ammoniated whey" produced by Moore, *et al.*, (Abstr. in J. Anim. Sci. 34:361-362, 1972) at Auburn University was used as a source of crude protein for lambs. The study indicated that the product was more effective as a protein supplement than urea but less effective than cottonseed meal. Subsequent studies by Alston, *et al.*, (Abstr. in J. Anim. Sci. 36:208, 1973) demonstrated that "ammoniated whey" could not be dried by conventional methods.

At the same time that the work described above was being carried out, pure ammonium salts of organic acids were also being evaluated as feed supplements for cattle by Allen, Henderson and Bergen at Michigan State University (1). The results of these studies encouraged Henderson to embark on a program of converting agricultural wastes to ammonium lactate. Henderson also demonstrated that a product analyzing 50% crude protein could be produced by fermentation of whey without sterilizing or pasteurizing the system.

In 1973 the project was extended cooperatively by the Department of Animal Husbandry and the Department of Microbiology and Public Health including the design and construction of a pilot plant. Approximately 500,000 lb of whey were processed by Henderson and Reddy to produce about 25 tons of condensed product for use in extensive beef cattle feeding trials (23). These feeding trials involved about 150 steers, and the experimental design was developed in cooperation with the Food and Drug

Administration to facilitate that agency's evaluation of the whey product as a feed additive. The fermented whey product performed nearly as well as soybean meal in the feeding trials when both were used as protein supplements (22). Alternate substrates are being evaluated by Reddy to expand the potential applications of the technology.

4.2 *Lactic Acid Fermentation*

Much of the fermentation technology of lactic acid production is directly applicable to the production of "ammoniated whey". Lactic acid was commercially produced by fermentation as early as 1881 (53), but in recent years the fermentation process has been partially displaced by one of direct chemical synthesis (48). Because of the previous importance of fermentation to the production of lactic acid, there are a considerable number of process descriptions (9, 48, 53, 56, 73), a review article (14) and a bibliography (13).

Commercial fermentation processes for the production of lactic acid utilize homofermentative lactic acid bacteria, which are capable of converting sugar to lactic acid with yields in excess of 95% (48, 53). Thermophilic, aciduric lactobacilli are particularly useful because they thrive at temperatures (45-50 C) which cannot be tolerated by most potential contaminants and because they tolerate high concentrations of acid in the medium. *Lactobacillus delbrueckii* is the organism of choice for fermenting sucrose- and dextrose-containing materials such as molasses and corn dextrose. *L. bulgaricus* is used to ferment lactose-containing substrates such as cheese whey.

Lactic acid bacteria synthesize very few of the vitamins, amino acids and unidentified growth factors required for their growth. In fact, these organisms are so fastidious that they are often used in biological

assays for various growth factors (15). In commercial processes utilizing lactic acid bacteria, it is often necessary to supplement the medium to insure that the fermentation proceeds at a reasonable rate (48).

Some of the limiting factors in the lactic fermentation were reported in 1928 by Rogers and Whittier (55). Although *Streptococcus lactis* was used as the test organism, many of the observations apply to lactobacilli as well (34, 35, 36). Control of the hydrogen ion concentration (pH) permits greater bacterial populations than when the accumulating acid is not neutralized. Even greater populations are attained when the culture with controlled pH is agitated with a mechanical stirrer, with air or with nitrogen, the effectiveness increasing in the order given. However, the concentration of undissociated acid is the principal factor in the limitation of growth and metabolism. A substance which is diffused through a semipermeable membrane, other than undissociated acid, also limits the growth of *Streptococcus lactis* (55).

More recently it was demonstrated that the yield as well as the rate of lactic acid production are functions of the pH of the fermentation. Up to an optimum value, the yield varies directly with pH; above the optimum, an inverse relationship exists (12, 20, 31).

A continuous process for the production of lactic acid from whey was developed by Whittier and Rogers (72). The process is unique in that sterilization of equipment and substrate are not required to prevent contamination by undesirable organisms. The fermentation temperature (45 C) and pH (5.0 to 5.8) as well as the use of lactobacilli are sufficient to inhibit the growth of undesirable organisms. The continuous process was practiced to a limited extent on a commercial scale, but problems with residual sugar in the final product hindered the recovery of lactic acid (9).

4.3 *Continuous Fermentation*

In general, continuous industrial processes are much more efficient than the corresponding batch processes (30, 69). This fact is adequately illustrated when one considers the chemical industry, in which most of the large-scale processes are operated continuously. Various authors report increases in productivity of five- to tenfold by converting batch fermentations to continuous fermentations (11, 40). This potential for very significant increases in productivity is reason enough to explore continuous fermentation when large-scale operations are anticipated.

The primary factor which contributes to the increased productivity of continuous fermentations is the marked reduction in processing time with equipment of the same holding capacity (42). A continuous fermentation also is more adaptable to instrumental control, is better integrated into the preceding and subsequent processing operations, and generally yields a more uniform product. Many advances are being made in the design of continuous fermentors. Some of the more novel approaches include plug-flow fermentors, tower fermentors, cyclone column fermentors (61) and dialysis fermentors (57).

There are two potential problems which are unique to continuous fermentation: contamination and culture degeneration (44). Contamination results from entry into the system of a fast-growing organism which eventually displaces the original population. Culture degeneration results from genotypic mutations and selection or from phenotypic adaptations of the original culture. While these problems of continuous fermentation have prevented its widespread application, they have not proven insurmountable. Many of the simpler fermentations such as the production of food and fodder yeast (42, 49), ethanol (25) and vinegar (4, 65) have benefited from the use of continuous processes. More recently, novel

processes for the production of single-cell protein from normal paraffins (58) and agricultural wastes (5) have also utilized continuous fermentations.

The successful introduction of a continuous process depends on a deeper knowledge of the process than is required for a batch process. Batch processes are often operated as an art based on empirical knowledge. Continuous processes, on the other hand, require a fundamental knowledge of the microbiology, biochemistry and total kinetics of the process (40).

To gain a more fundamental understanding of fermentation processes, it has been beneficial to describe the processes in terms of mathematical models. Equations have been developed which describe the relationship of throughput, microbial propagation, product formation, substrate utilization and the like. Due to the complexity of biological systems it is seldom possible to obtain a strict mathematical model of all the factors involved. Many excellent reviews discuss the theoretical analysis of continuous culture systems (11, 18, 43, 46, 54).

Given a valid mathematical model for a continuous fermentation, it is possible to simulate the fermentation on an analog or a digital computer. It is possible to complete a computer-simulated fermentation in minutes whereas the actual fermentation may take hours or even days. Herein lies the value of the simulation: many process variables can be evaluated on the computer in a fraction of the time it would take to actually conduct the experiments. Furthermore, the cost of computer time is generally much less than the cost of the materials, equipment and labor used in the actual experiment. The extent to which an actual fermentation is predicted by a computer simulation will depend on how closely the mathematical model reflects reality (57).

5. MATERIALS AND METHODS

5.1 *Bacterial Culture*

Lactobacillus bulgaricus strain 2217 (Chris Hanson's Laboratory, Milwaukee, Wis.) was used throughout this study. This organism was selected by Reddy (23) on the basis of its high rate of acid production in the pH range of 5.0 to 6.0. The culture was maintained in a sterile medium of 10% skim milk powder and 90% tap water contained in 25-ml screw-top test tubes. At least every two weeks the culture was transferred to fresh medium. Inoculated tubes were incubated 18 to 24 hr at 44 C. Coagulation of the milk served as a positive test for bacterial growth. After incubation the cultures were stored at 4 C.

5.2 *Substrate*

Both fresh and reconstituted cottage cheese whey were used as fermentation substrates. Both types of whey were obtained from Michigan Milk Producers Association, Ovid, Michigan. It was determined experimentally that there was no difference in the two types of whey when either was used as the fermentation substrate. For the continuous fermentations, some difficulty was experienced initially in getting the powdered whey into solution. However, the following procedure was helpful in minimizing the problem.

Three pounds of whey powder were put into a 5-gal polyethylene carboy along with 35 lb of water. The water and powder were partially mixed by putting the carboy on its side and rocking it for about a minute. This procedure wetted most of the powder, but many small lumps were still present. The carboy was cooled (4 C) for 24 hr during which time the

lumps dissolved. Before use, the contents of the carboy were again mixed by rocking the carboy.

5.3 *Fermentation Equipment*

All fermentations were carried out in a 14-liter bench-top fermentor with automatic temperature control (Model MA140F1, Fermentation Design, Allentown, Penn.). The pH was controlled by an automatic pH control module (Model pH-22, New Brunswick Scientific Co., Inc., New Brunswick, N. J.).

Continuous fermentations were conducted by pumping reconstituted whey from an unsterilized feed reservoir to the fermentor and allowing the product to overflow into a product reservoir. The feed reservoir was a 5 gal, polyethylene carboy. This reservoir along with its contents were chilled (4 C) for at least one day before using. While in use, the reservoir was insulated with a heavy blanket, which was sufficient to prevent the temperature of the whey from exceeding 16 C in 24 hr.

The feed rate to the fermentor was controlled by a finger-type, peristaltic pump (Model T8, Sigmamotor Company, Middleport, N. Y.). Polyurethane tubing, obtainable from the pump manufacturer, was required for the section of line that passed through the pump; natural rubber tubing split in less than 48 hr, and Tygon tubing took a permanent "set" that resulted in a variable flow rate.

A simple overflow device was developed to maintain a constant level in the fermentor (Fig. 1). A piece of 1/4-in stainless steel tubing was coupled to one of the fermentor top fittings by means of a piece of 1/4-in O.D. natural rubber tubing. The stainless steel tubing was cut to a length that determined the liquid level in the fermentor. A small amount of CO₂ was continuously purged into the fermentor and escaped through the

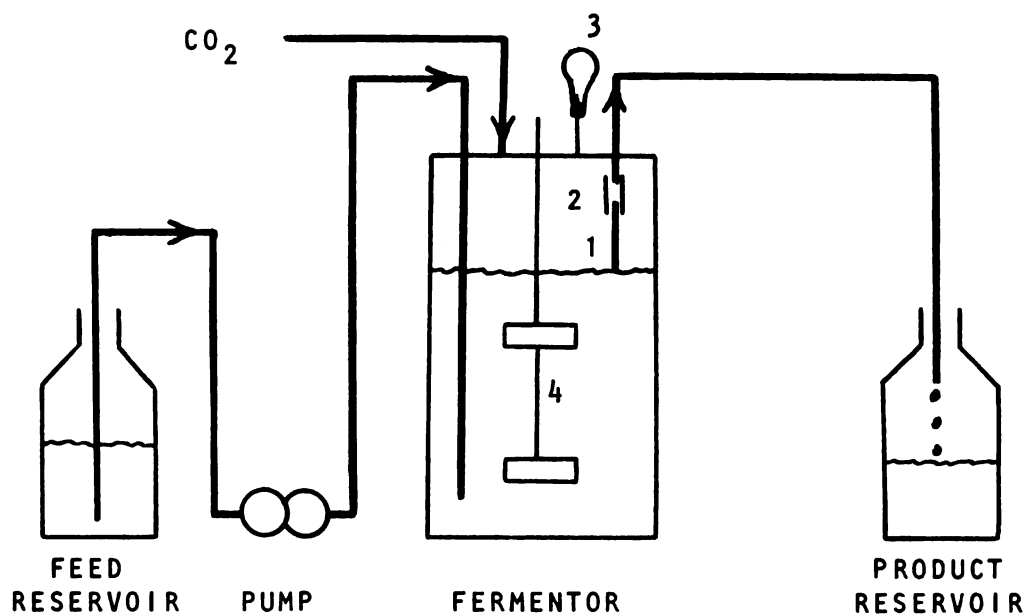


FIG. 1. *Schematic diagram of level-control device. Symbols: 1, stainless steel tubing; 2, rubber coupling; 3, balloon; 4, agitator.*

piece of stainless steel tubing, the only open port. A toy balloon taped to one of the fermentor top fittings served as a pressure release device in the event the outlet became plugged. As the liquid rose and covered the end of the stainless steel tubing, the CO_2 purge developed a slight pressure within the fermentor. The pressure was sufficient to force the liquid up and out of the fermentor. As the liquid was forced out, the liquid level dropped until CO_2 could again escape through the stainless tubing. The liquid level fluctuations were so minor as to be undetectable to the casual observer. The liquid volume in the fermentor was 9.6 liters unless otherwise noted.

Batch fermentations were conducted by inoculating 10 liters of unsterilized whey (pH 5.5, 44 C) with 700 ml of the pure bacterial culture. Reconstituted whey was prepared for the batch fermentations by adding sufficient tap water to 800 g of powdered whey to bring the liquid level in

the fermentor to 10 liters. The pH was maintained at 5.5 ± 0.1 by the automatic addition of anhydrous ammonia.

5.4 *Procedure for Continuous Fermentation*

To start, the continuous fermentor was charged with 10 liters of unsupplemented, reconstituted whey, the temperature and pH were adjusted to the desired levels, and 700 ml of inoculum were added. The fermentation was allowed to proceed batch-wise until most of the lactose was fermented (approximately 24 hr). At that time the continuous feed was started. Samples were taken approximately every 12 hr, and the fermentation was allowed to proceed at least 48 hr before changing to a new set of operating conditions. Other conditions were evaluated by changing the appropriate parameters and allowing the fermentation to re-establish a new steady state.

Normally, the feed rate was adjusted to give the reported retention times. However, to achieve the retention time of 7.6 hr in one stage and 15.2 hr in two stages, the liquid volume in the fermentor was reduced to 4.9 liters; and the feed rate was adjusted accordingly.

The continuous fermentor was in service for 42 days, when it was terminated voluntarily. The system was interrupted only for one weekend, at which time the fermentor and its contents were stored at 4 C. The fermentor was reinoculated at the time that it was put back in service, but this precautionary measure may not have been necessary.

5.5 *Fermentor Measurements*

The pH of a sample from the fermentor was checked on a separate pH meter which was calibrated against buffers of known pH. The pH of the

sample was used to calibrate the pH control unit at least twice daily. The maximum error for the pH control system was estimated to be ± 0.1 pH unit.

A thermometer inserted into the thermal well in the top of the fermentor was used to set the temperature control point. The temperature controller maintained the temperature within ± 0.5 C of the set point.

The overflow rate from the fermentor was determined twice daily by measuring the collected product with a 2-liter graduated cylinder. The flow rate varied less than 3% during the 12-hr collection period and was used to calculate average retention times.

Samples were withdrawn through the top of the fermentor by means of a 25-ml pipette. The samples were quickly placed in the freezing compartment of a domestic refrigerator to stop further fermentation and held there until the time of analysis.

5.6 *Lactose Determination*

Lactose determinations were made by a modification of the picric acid method of Perry and Doan (51). One ml of sample was pipetted into a 250-ml Erlenmeyer flask and diluted with 99 ml of saturated picric acid. If the sample was likely to contain less than 2% lactose, only 49 ml of saturated picric acid were used; and the final result was divided by 2.

The flask contents were shaken and filtered (#588 filter paper, Schleicher & Schuell, Keene, New Hamp.). Two ml of the filtrate were transferred to a 20 X 150 mm culture tube (which had been previously marked at the 20 ml level) containing 1.0 ml of Na_2CO_3 solution (25 g per 100 ml). The tube was stoppered lightly, shaken and placed in a boiling water bath for 20 ± 0.5 min. The contents of the tube were then cooled to approximately 20 C in a water bath, diluted to 20 ml with distilled

water and mixed by inverting. A portion was transferred to a colorimeter tube, and a reading was obtained at 520 nm within 20 min of removal from the boiling water bath.

A blank consisting of 2.0 ml of saturated picric acid and 1.0 ml of Na_2CO_3 solution was heated, cooled and diluted along with the unknowns for adjustment of the zero point of the colorimeter (Model 20, Bausch and Lomb, Rochester, N. Y.). A standard solution was made by diluting 5.0 g of dry lactose to 100 ml in a volumetric flask. Aliquots of 0.2, 0.5 and 1.0 ml of this solution were analyzed and the results were used to construct a standard curve.

In no fermentation samples was an apparent lactose concentration of less than 0.2% obtained. It was suspected, therefore, that a nonspecific background color was present in the fermentation samples and was not present in the pure lactose samples used for constructing the standard curve. Four samples which had been reported as 0.2% lactose were submitted to the Department of Food Science and Human Nutrition at Michigan State University for lactose analysis by the A.O.A.C. method (26). Two of the samples contained no trace of lactose and the other two contained only a trace (less than 0.1%). These analyses confirmed that a nonspecific background color was in fact present in the fermentation samples. Consequently, the lactose results were corrected for this background color by subtracting 0.2% lactose from all samples which were analyzed with 49 ml picric acid and subtracting 0.1% lactose from all samples analyzed with 99 ml picric acid.

Residual lactose was the preferred (even though indirect) indicator of rate and extent of fermentation because there was less variance in the lactose determinations than in the lactic acid determinations and because

the endpoint of the fermentation was indicated more precisely by lactose concentration than by lactic acid concentration.

5.7 Ammonium Ion Determination

The ammonium ion concentration in the medium was used as an index of lactic acid production and was determined by a modification of a colorimetric method reported by Johnson (29). One ml of a sample containing 4 to 40 μg per ml of ammonium ion was pipetted to a spectrophotometer cuvette. To the cuvette were added 2.0 ml of Nessler's reagent and 3.0 ml of 2N NaOH. The Nessler's reagent contained (per liter) 4.00 g of KI, 4.00 g of HgI_2 and 1.75 g of gum ghatti. The contents of the cuvette were mixed by inverting and allowed to develop color at room temperature for 15 min. The absorbance was read at 490 nm. Blanks contained 1.0 ml of distilled water in place of the 1.0 ml sample.

The standard for the ammonium ion determination was an $(\text{NH}_4)_2\text{SO}_4$ solution containing 100 μg ammonium nitrogen per ml. To prepare a standard curve, 0.1, 0.3, 0.5, 0.7 and 1.0 ml of the standard and sufficient water to bring the volume to 1.0 ml were added to cuvettes in place of the 1.0 ml sample.

5.8 Lactic Acid Determination

Lactic acid was analyzed by a simplified gas chromatographic procedure specifically and recently developed for bacterial metabolic products (8). A 1.0 ml sample of the culture was drained through 1 ml of cation-exchange resin (Dowex 50W-X8, 50-100 mesh, H-form, washed in water; BioRad Laboratories, Richmond, Calif.) on glass wool in a Pasteur pipette. After the sample drained through the resin, the resin was

washed twice with 0.5 ml distilled water. All the fluid from the pipette was collected, and an aliquot was directly analyzed in a gas chromatograph (Model 810, hydrogen flame detector, F&M Scientific Corp., Avondale, Pa.). A 1.8 m by 2 mm I.D. coiled glass column (Ansbec Corp., Ann Arbor, Michigan) was packed with a porous polymer (Chromosorb 101, 80/100 mesh, Johns-Manville, Denver, Colo.). The column was conditioned overnight at 250 C and then run isothermally at 220 C. The inlet temperature was 250 C, and the detector temperature was 230 C. A 2.0 μ l sample was injected. The carrier gas was 10 ml per min of nitrogen, and the hydrogen and air pressures were 7.5 and 11.0 psig, respectively. Solutions of 1.1, 2.2, 4.3 and 6.0% lactic acid were analyzed, and the results were used to construct a standard curve.

6. THEORY

A model specifically describing growth and product formation in the microbial production of lactic acid was developed by Luedeking (37), and this model is reviewed and modified below. This same model was published by Luedeking and Piret in a primary scientific publication (38, 39). Some of the data referred to as "Luedeking's data" in this thesis also may be found in the primary publication.

A simple continuous fermentor is represented by the generalized model known as a continuous stirred tank fermentor (Fig. 2). It is

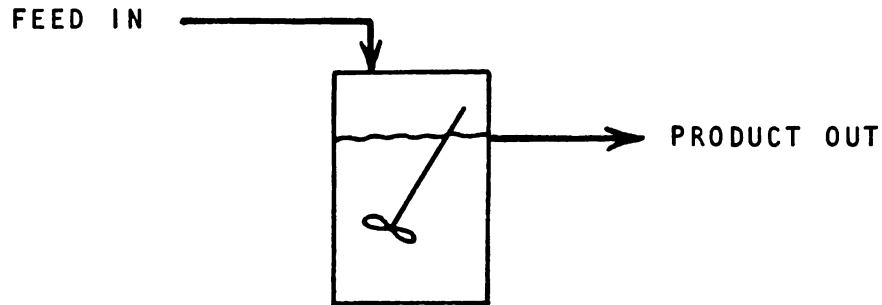


Fig. 2. *Continuous stirred tank fermentor*

assumed that such a fermentor is sufficiently mixed so that the composition of the effluent is the same as the contents of the fermentor. A material balance of the bacterial mass of the system can be stated as:

$$\begin{array}{ccccccc} \text{Rate of} & & \text{Rate of} & & \text{Rate of} & & \text{Rate of} \\ \text{Feed} & + & \text{Production} & = & \text{Withdrawal} & + & \text{Accumulation} \end{array}$$

Algebraically, the material balance is stated as:

$$FX_o + V(dX/d\theta)_G = FX + V(dX/d\theta) \quad (1)$$

where: F = rate of continuous feed, volume per unit time

V = operating volume of liquid in the fermentor

X = concentration of bacterial mass in the fermentor

X_o = concentration of bacterial mass in the feed stream

θ = time

$(dX/d\theta)_G$ = rate of change in concentration of bacterial mass due to growth, concentration per unit time.

In Equation 1, $(dX/d\theta)_G$ is used to distinguish the rate of change in concentration of bacterial mass that is due to growth from the rate of change that is due to all other factors. $(dX/d\theta)$ represents the net rate of change from all factors.

The specific growth rate k is defined as the rate of change in bacterial mass per unit of bacterial mass present in the system:

$$k = (dX/d\theta)_G / X \quad (2)$$

Dividing Equation 1 by V and substituting r for F/V results in the following equation:

$$rX_o + (dX/d\theta)_G = rX + dX/d\theta \quad (3)$$

where: r = throughput rate, volume of feed per unit time per unit operating volume.

The reciprocal of r is the retention time of the average cell within the fermentor. When the feed is sterile (i.e., when $X_o = 0$) and the specific growth rate k is substituted into Equation 3, it becomes:

$$dX/d\theta = (k - r)X \quad (4)$$

Under steady state conditions there is no change in the concentration of bacterial mass as a function of time (i.e., $dX/d\theta = 0$). Therefore, the throughput rate is equal to the specific growth rate constant (i.e., $r = k$). This relationship is the basis for design of the classical Chemostat (or Bactogen) described by Monod (61). In the Chemostat, growth is limited by one of the nutrients or metabolic products. Consequently, the bacterial population adjusts itself until the specific growth rate is equal to the throughput rate.

It is often desirable to relate the rate of product formation to some condition in the fermentor. Generally, the concentration of bacterial mass is used for this purpose. It has been empirically demonstrated that in lactic acid fermentations the rate of change of product concentration is a function of both the concentration of bacterial mass and also the rate of growth of the bacterial mass (37):

$$(dP/d\theta)_G = \alpha(dX/d\theta)_G + \beta X \quad (5)$$

where: P = concentration of product

$(dP/d\theta)_G$ = rate of change in concentration of product
due to growth, concentration per unit time

α, β = constants of proportionality fixed by the
organism, substrate, pH and temperature.

Since the specific growth rate is given by $(dX/d\theta)_G/X$, Equation 5 becomes:

$$(dP/d\theta)_G = (\alpha k + \beta)X \quad (6)$$

Therefore, a material balance for the product can be written as follows:

$$dP/d\theta = (\alpha k + \beta)X - rP \quad (7)$$

For homofermentative lactic acid fermentations it can be assumed with reasonable accuracy that the rate of product formation is proportional to the rate of substrate utilization (12, 20, 37), because only a small, relatively constant fraction of the substrate is incorporated into bacterial mass (20). Therefore:

$$(dP/d\theta)_G = -Y(dS/d\theta)_G \quad (8)$$

where: S = concentration of substrate

$(dS/d\theta)_G$ = rate of substrate utilization by the bacterial culture

Y = yield constant expressed as the ratio of product formed to substrate consumed.

A material balance for the system again yields an expression for the rate of change in the substrate:

$$dS/d\theta = (S_0 - S)r - (\alpha k + \beta)X/Y \quad (9)$$

where: S_0 = concentration of substrate in the feed.

In summary, the material balance equations for bacterial mass, product and substrate in continuous lactic acid fermentations are:

$$dX/d\theta = (k - r)X \quad (4)$$

$$dP/d\theta = (\alpha k + \beta)X - rP \quad (7)$$

$$dS/d\theta = (S_0 - S)r - (\alpha k + \beta)X/Y \quad (9)$$

It should be noted that no assumption has been made for a constant value for k as was done previously (37). As a result, the above equations should hold both for the logarithmic growth phase where k is a constant and for the phase in which an accumulation of metabolic products causes a decline in the value of k .

To express the specific growth rate as a function of the product concentration, assume that the following linear relationship exists:

$$k_i = k_{\max}(1 - P/P_{\max}) \quad (10)$$

where: k_i = specific growth rate for a given product concentration if sufficient substrate were present.

$k_{\max}$ = maximum specific growth rate attained in the fermentation.

$P_{\max}$ = product concentration at which k_i first equals zero.

Equation 10 is a simple relationship between k_i and $k_{\max}$ which adequately describes the data of Luedeking in the pH range of 4.8 to 5.6 (37).

By inspection, one can see that Equations 4, 7 and 9 are not valid when the substrate has been exhausted. Equation 4 would predict an increase in bacterial mass even when there was no substrate present. Similar problems can be observed in Equations 8 and 10.

In enzyme kinetics, the Michaelis-Menten equation is used to relate the enzyme reaction rate to the concentration of substrate:

$$v = \frac{V_{\max} S}{K_s + S} \quad (11)$$

where: v = enzyme reaction rate

$V_{\max}$ = maximum enzyme reaction rate if substrate were not limiting.

K_s = saturation constant

Monod found that bacterial growth was analogous to an enzyme reaction (11). Therefore the reaction rate can be described as a function of substrate and metabolic products by incorporating Equations 10 and 11 into

Equation 4 to give:

$$\frac{dX}{d\theta} = \left[k_i \left(\frac{S}{K_s + S} \right) - r \right] X \quad (12)$$

In Equation 12, k_i is analogous to $V_{\max}$ in Equation 11. In Equation 7, both αk and β are reaction velocities which must be dependent on substrate concentration.

Again, use of Equation 10, analogy with Equation 11 and application of both to Equation 7 give:

$$\frac{dP}{d\theta} = (\alpha k_i + \beta) \left(\frac{S}{K_s + S} \right) X - rP \quad (13)$$

Similarly, Equation 9 becomes:

$$\frac{dS}{d\theta} = (S_o - S)r - (\alpha k_i + \beta) \left(\frac{S}{K_s + S} \right) \frac{X}{Y} \quad (14)$$

Therefore, the revised material balance equations which take into account a variable specific growth rate constant and substrate exhaustion are:

$$\frac{dX}{d\theta} = \left[k_i \left(\frac{S}{K_s + S} \right) - r \right] X \quad (12)$$

$$\frac{dP}{d\theta} = (\alpha k_i + \beta) \left(\frac{S}{K_s + S} \right) X - rP \quad (13)$$

$$\frac{dS}{d\theta} = (S_o - S)r - (\alpha k_i + \beta) \left(\frac{S}{K_s + S} \right) \frac{X}{Y} \quad (14)$$

By inspection one can now see that Equations 12, 13 and 14 could be valid even when the substrate is exhausted. As S approaches 0, the quantity $S/(K_s + S)$ must also approach 0, indicating that no more change can take place by bacterial metabolism.

7. RESULTS

7.1 *Computer Simulation of Batch Fermentation*

The material balance equations (Equations 12, 13 and 14) served as the basis for the computer simulation, which was programmed on a digital computer by Dr. George Coulman of the Department of Chemical Engineering, Michigan State University. The purpose of the simulation was to guide the experimental part of the research program. Therefore, it was necessary to execute the program before the experimental results were available. However, some first approximations were required for the constants in the material-balance equations. These initial values were taken from the data of Luedeking (37) with full realization that different media, cultures and neutralizing agents were used in the two systems. However, these differences were inconsequential since the purpose of this phase of the programming was to indicate general trends and not to yield absolute values.

To verify that Equations 12, 13 and 14 and the constants were in fact valid, they were first used to simulate Luedeking's data for batch fermentation at pH 5.6. The program was started at Hour 3 to avoid the problems associated with simulating the lag phase. This was no compromise in the original objectives since a lag phase does not occur in continuous culture. The values of the various constants and the initial conditions are given in Table 1.

The specific growth rate constant k_1 given by Luedeking varied throughout the fermentation (Fig. 3). In the pH range of 4.8 to 5.6, k_1 was approximately proportional to the product concentration. Therefore, Equation 10 was used to approximate the curve for pH 5.6. For $P > 38$,

TABLE 1. *Values used in the simulation of batch fermentation,
from Luedeking (37)*

Symbol	Value	Reference
α	2.2	(37), p. I-24
β	0.49	" "
P (initial)	1.1	" Table I-1, Hour 3
S (initial)	50.	" " "
X (initial)	0.283	" " "
for $P \leq 38$:		
$k_{p_{\max}}$	0.48	Fig. 3 in this thesis
$p_{\max}$	50.	" " " " "
for $P > 38$:		
$k_{p_{\max}}$	1.1	" " " " "
$p_{\max}$	43.	" " " " "
r	0.	(Batch fermentation)

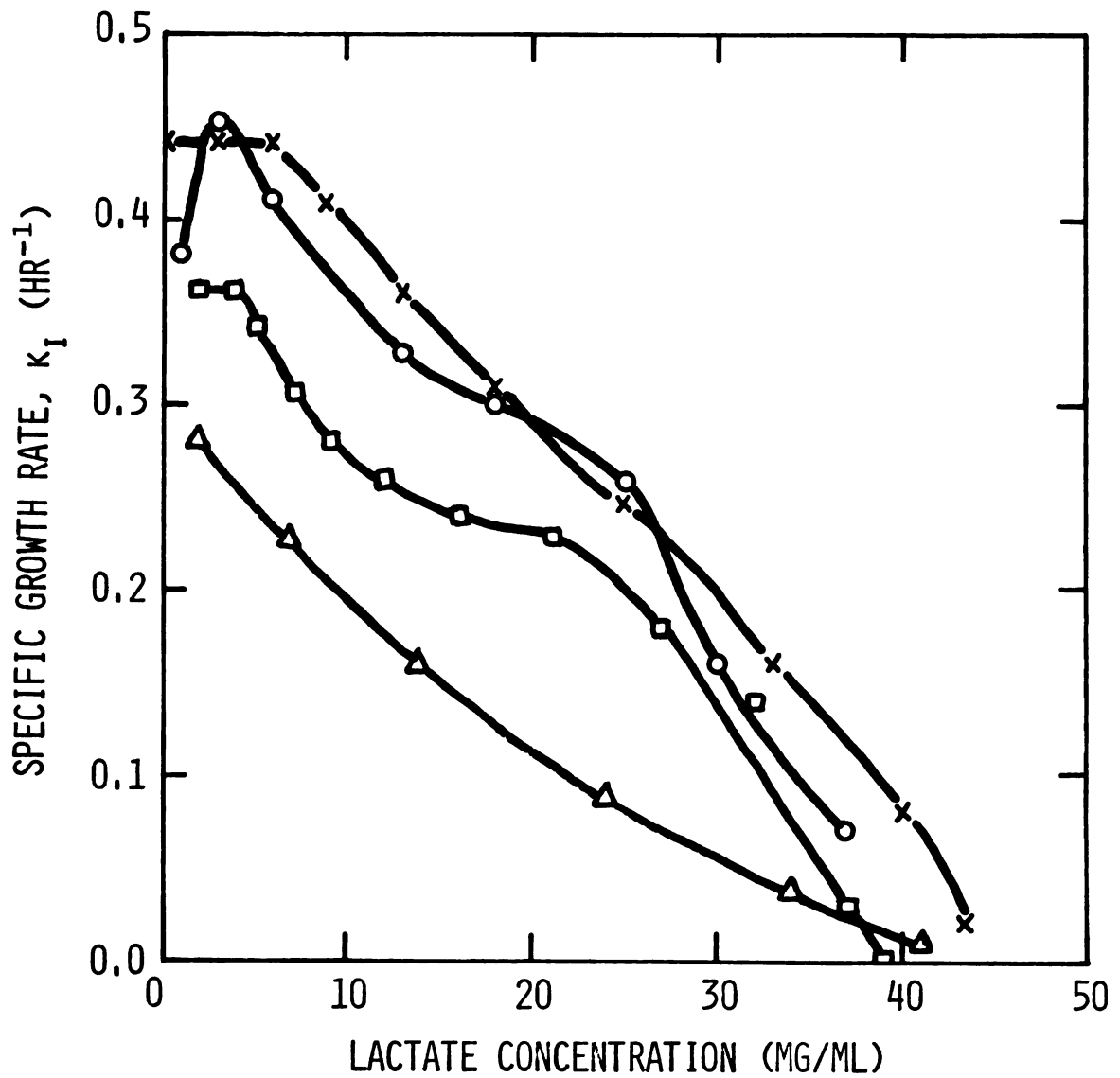


FIG. 3. Effect of lactate concentration of the specific growth rate, with data from Luedeking (37). Symbols: x, pH 5.6; O, pH 5.4; ■, pH 5.2; Δ, pH 4.8.

the slope of the curve became more negative; therefore, the values for $k_{\max}$ and $P_{\max}$ were reset to reflect this change (Table 1).

The mathematical model (Equations 12, 13 and 14) was shown to simulate closely Luedeking's batch fermentation data (Fig. 4). Near the end of the fermentation (i.e., Hour 13 to 14), the simulated bacterial density deviated from the experimental bacterial density because the simulated product concentration was slightly less than the experimental product concentration. Therefore in the simulation, a small amount of substrate remained after 13 hr to support bacterial growth.

7.2 *Simulated and Experimental Continuous Fermentation*

After Luedeking's batch fermentation data were found to verify the mathematical model, the same model was modified and used to simulate single- and multi-stage continuous fermentations. As indicated in Table 1, the term r in Equations 12, 13 and 14 was set to 0 for the simulation of a batch fermentation. However, this term was retained when simulating a continuous fermentation. In simulating a multi-stage continuous fermentation, the product from one stage was used as the feed for the subsequent stage. Equations 12 and 13 were then modified to take into account the fact that an inlet stream to a given stage could contain appreciable amounts of product and bacterial mass. Equation 14 previously contained a term for the substrate concentration in the inlet stream. Therefore, the three generalized material-balance equations for any stage of a multi-stage fermentor are:

$$\frac{dX}{d\theta} = \left[k_i \left(\frac{S}{K_s + S} \right) - r \right] X + rX_o \quad (15)$$

$$\frac{dP}{d\theta} = (\alpha k_i + \beta) \left(\frac{S}{K_s + S} \right) X + r(P_o - P) \quad (16)$$

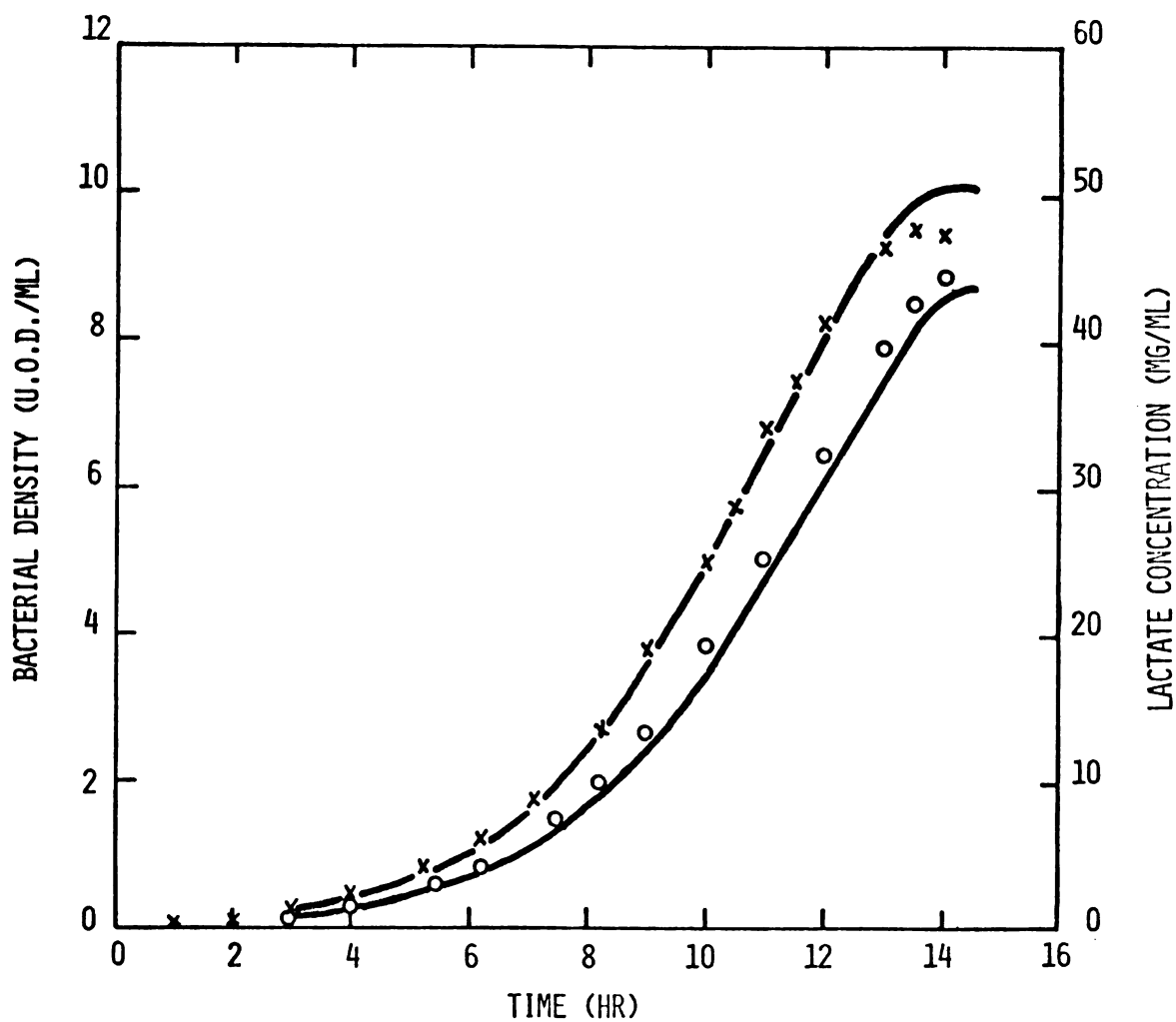


FIG. 4. Comparison of curves from the computer simulated data with the experimental data of Luedeking (37) for bacterial density (X) and lactate concentration (O).

$$\frac{dS}{d\theta} = (S_o - S)r - (\alpha k_i + \beta) \left(\frac{S}{K_s + S} \right) \frac{X}{Y} \quad (17)$$

The concentrations X_o , P_o and S_o refer to the concentration of the bacterial mass, product and substrate, respectively, in the inlet stream to a given stage of a fermentor. Equations 15, 16 and 17 were used to simulate continuous fermentors with one, two and three stages. As in the batch fermentation, Luedeking's data at pH 5.6 was used to define k_i at various product concentrations (Equation 10 and Table 1). The feed to the multi-stage continuous fermentors contained 50 mg per ml glucose, no bacteria and no product.

The results of the simulation (Fig. 5) indicated the general trends one could expect by varying the retention time and by using a multi-stage fermentation. The *total* retention time for all stages is given in Fig. 5. The retention time for each stage can be calculated by dividing the total retention time by the number of stages. The ordinate in Fig. 5 refers to the amount of glucose in the effluent from the last stage. As the retention time in a simulated, single-stage fermentation was increased, the concentration of residual sugar decreased. A point (at approximately 10 hr) was reached at which only marginal improvements were achieved by further increasing the retention time. Further reductions in the concentration of sugar were achieved by separating the fermentation into two and three stages. However, three stages gave only marginal improvement over two stages.

The experimental results for the continuous lactic acid fermentation of reconstituted whey (Fig. 6) were similar to those for the simulated glucose fermentation (Fig. 5). The experimental fermentation at pH 5.5 reached a point (at approximately 15 hr) at which a further increase in

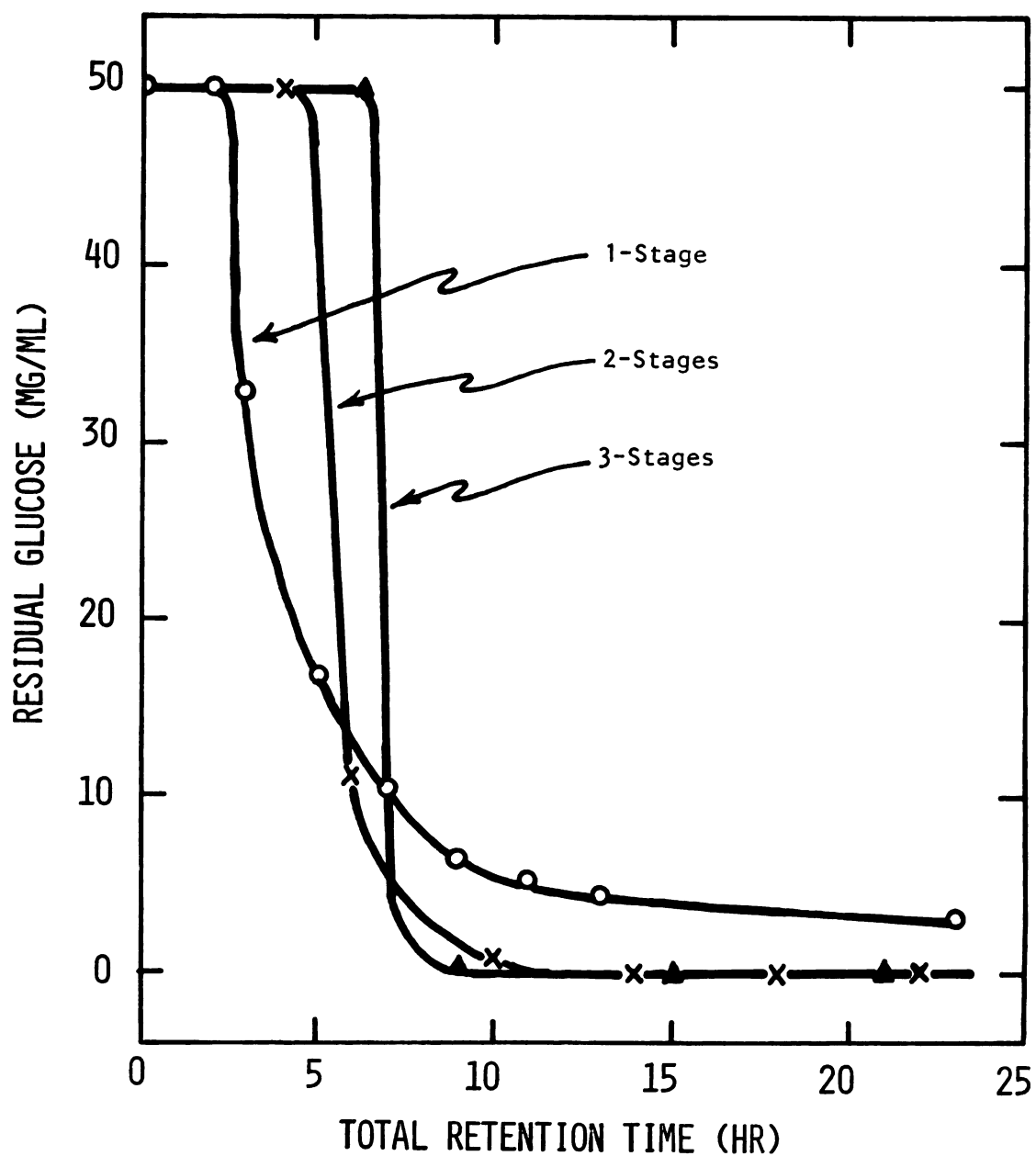


FIG. 5. Effect of total retention time in single- and multi-stage fermentations as predicted by simulation. Symbols: $\circ$, one stage; $\times$, two stages; $\blacktriangle$, three stages.

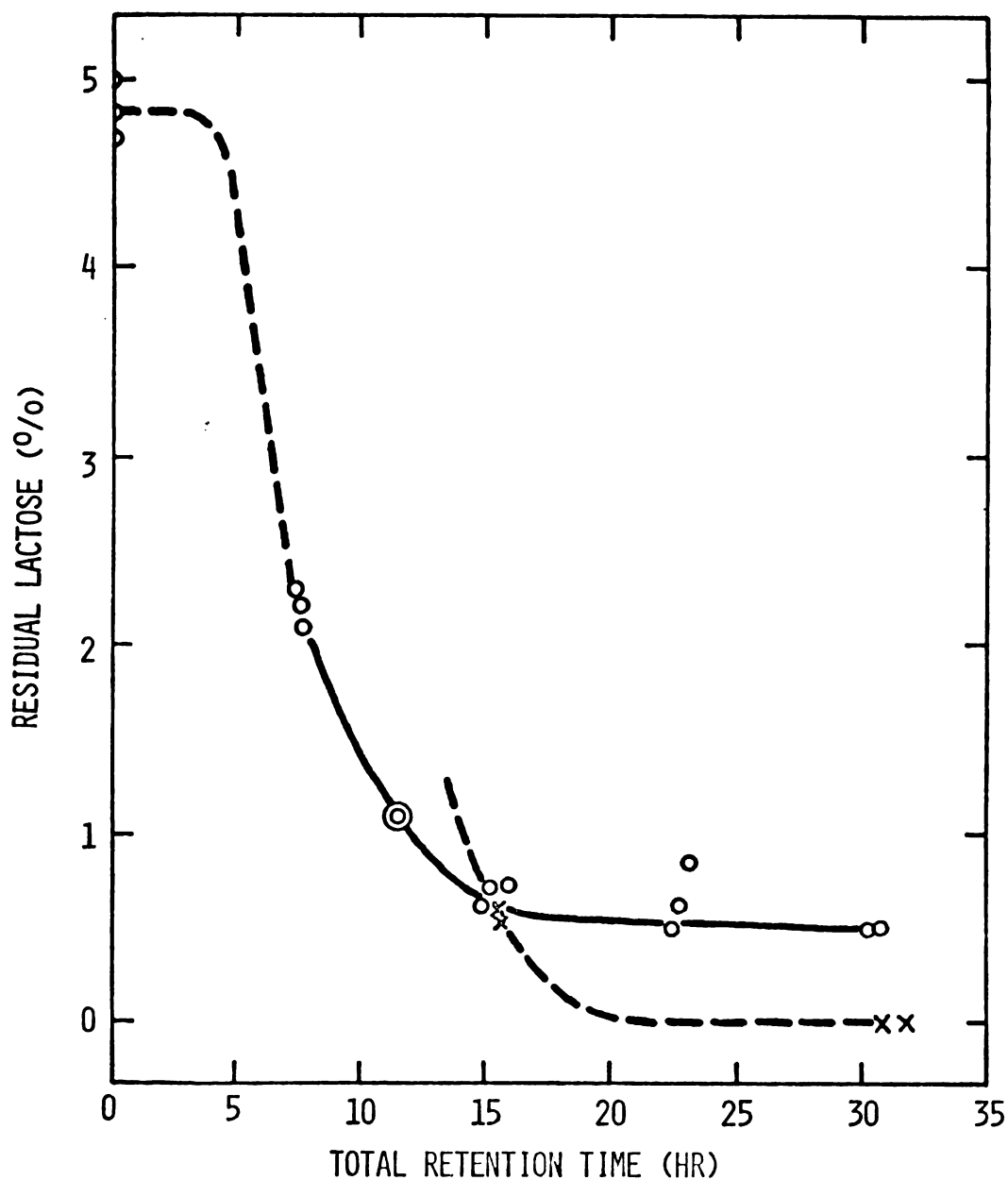


FIG. 6. Effect of the total retention time and staging on the level of residual lactose in continuous fermentation of reconstituted whey. The results shown in Fig. 5 were used to interpolate the curve for the single-stage (O) between 0 and 7 hr and for the double-stage (X) between 12 and 31 hr.

the retention time caused essentially no further reduction in the residual lactose concentration. However, the use of two stages significantly reduced the level of residual lactose. In fact, it was possible to achieve more than 98% lactose conversion by using two stages with a retention time of 15.5 hr in each stage (i.e., a total retention time of 31 hr).

Sufficient information is given in Fig. 6 to extend the mathematical model to the whey fermentation. One can obtain k_i as a function of P (Fig. 7) by recalling that k_i is equal to the reciprocal of the retention time under steady state conditions as long as S is much greater than K_s (Equation 12). Therefore for the whey system under steady-state conditions (i.e., $dX/d\theta = 0$), there were no unknowns in Equation 12. Since α and β are not independent, Equation 13 contained only two unknowns: $(\alpha k_i + \beta)$ and X . Hence, Equation 13 (and similarly Equation 14) was solved for X by assigning an arbitrary value to α and adjusting β by successive approximations to fit the model to the experimental data.

The previous values of 2.2 and 0.49, respectively, were used as first approximations for α and β . By successive approximations, values of $\alpha = 2.2$ and $\beta = 0.2$ were found to yield a good fit of the model to the experimental data (Fig. 8). Therefore, it was possible to extend the model to include the continuous fermentation of whey. The model then enabled one to predict the performance of a single- or multi-stage fermentor operating over a wide range of retention times.

7.3 *Adaptation of the Continuous Culture*

At the end of the 42 day continuous fermentation, the bacterial culture reduced the lactose concentration to a lower level than at the start. This phenomenon is illustrated by the data in Table 2. In the early part of the fermentation, with a pH of 5.5 and a retention time of 22 hr, the

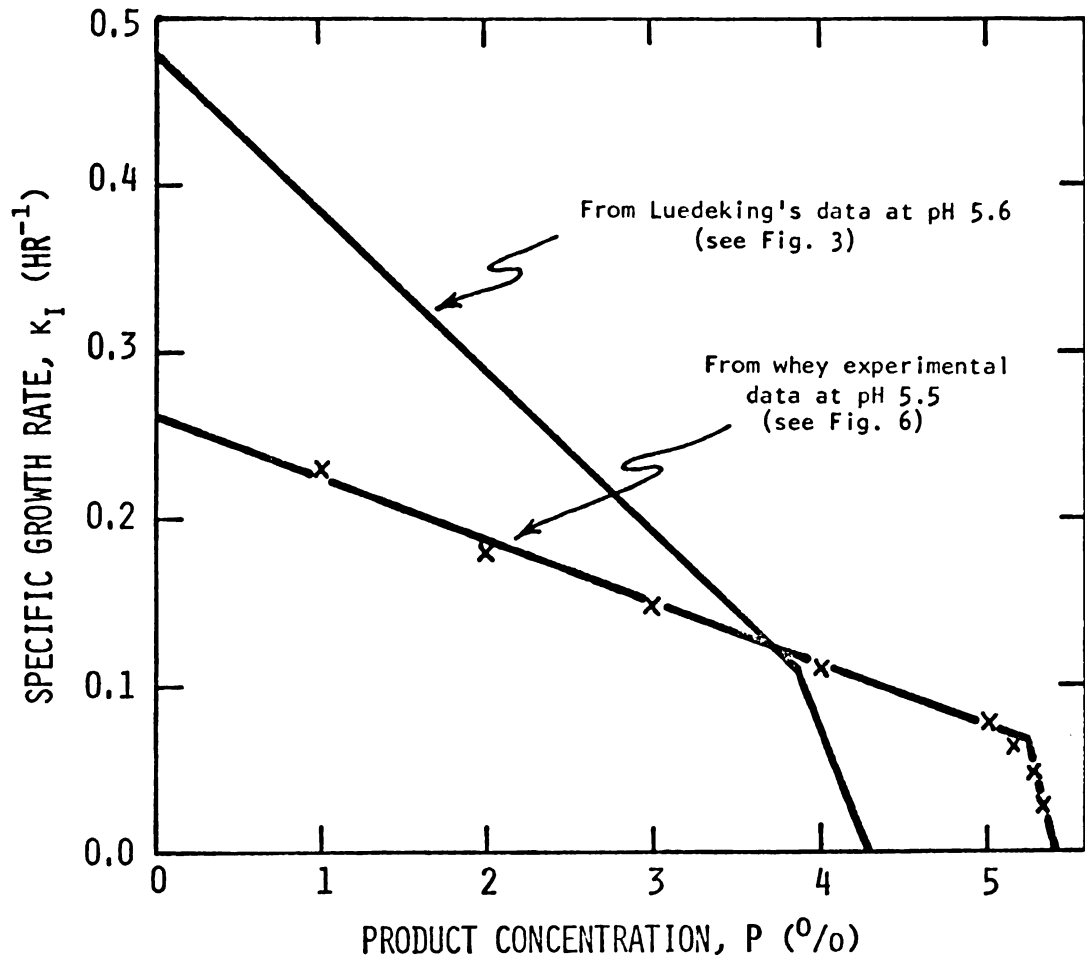


FIG. 7. Graphs of the linear approximation used to simulate the effect of product concentration on the specific growth rate with calculated data points indicated (X).

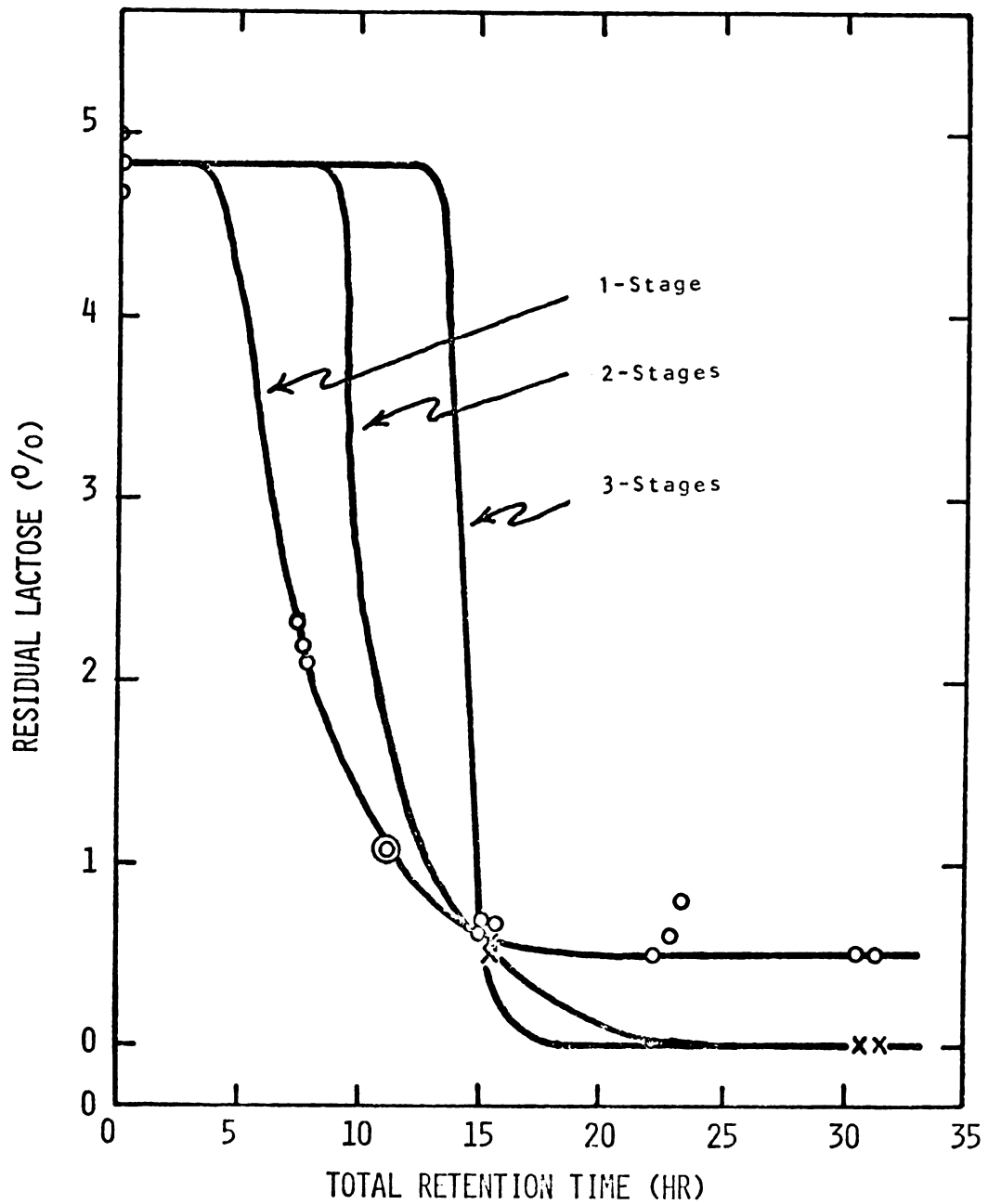


FIG. 8. Effect of the total retention time and staging on the level of residual lactose in whey as predicted by computer simulation (curves). Experimental data points for single-stage (O) and double-stage (X) fermentations are included for comparative purposes.

TABLE 2. *Adaptation of bacterial culture as indicated by a change in residual lactose concentration during prolonged continuous fermentation at two levels of pH*

pH	Day	Retention Time (hr)	Residual Lactose (%)
5.5	2	22.0	1.9
	3	21.6	1.7
	11	21.4	1.6
	12	21.6	1.6
	39	22.2	0.5
	40	22.6	0.6
5.8	6	22.0	1.0
	6	22.2	1.0
	17	15.7	0.9
	17	14.7	0.8
	18	14.5	0.7
	19	11.7	0.6
	19	12.0	0.7

culture reduced the lactose level in the whey to about 1.7%. A month later, at the same operating conditions, the culture reduced the lactose level to about 0.6%.

The steady improvement in performance also occurred at pH 5.8. Although the retention time was reduced over a 13 day period, the percentage of lactose in the product was also reduced during that time period, in contrast to the results presented in Fig. 6.

A qualitative observation also indicated a change in the bacterial culture. The *L. bulgaricus* #2217 culture used to inoculate the continuous fermentor on Day 1 had a pronounced, yoghurt-like odor. However, an inoculum prepared from a freeze-dried sample of the continuous fermentor culture taken on Day 35 had very little odor of any kind.

The data in Table 2 were used to identify the period from Day 14 to Day 19 as a period of significant change in the qualitative ability of the culture to ferment lactose to lactic acid. Therefore, no data from this time period were used to illustrate the effect of pH or retention time on the conversion of lactose.

7.4 *Effect of pH in the Range of 5.5 to 6.0 on Continuous Fermentation*

Increasing the pH of the continuous fermentation from 5.5 to 6.0 resulted in a significant reduction in the amount of residual lactose in the final product. The data in the upper portion of Table 3 show the effect of pH before the adaptation of the bacterial culture. Since there was no significant difference in the retention times (in the upper portion of the table) the difference in levels of lactose was due to the pH effect. A 37% reduction in the amount of residual lactose (from 1.70% to 1.07%) occurred when the pH was raised from 5.5 to 5.8. Further increasing the pH to 6.0 only reduced the residual lactose concentration an additional 7% (from 1.07% to 0.95%).

TABLE 3. *Effect of pH in the range of 5.5 to 6.0 as indicated by residual lactose concentration*

Period	pH	Residual lactose (%)		Retention time (hr)	
		Replication	Average	Replication	Average
Before Adaptation	5.5	1.9	1.70	22.0	21.3
		1.7		21.6	
		1.6		21.4	
		1.6		21.6	
	5.8	1.0	1.07	22.0	
		1.0		22.2	
	6.0	0.9	0.95	20.6	
		1.0		20.3	
After Adaptation	5.5	0.7	0.70	15.7	15.2
		0.7		15.4	
		0.8		15.2	
		0.7		15.2	
		0.6		15.2	
	6.0	0.3	0.30	14.8	
		0.3		15.1	
	5.5	1.1	1.10	11.5	11.3
		1.1		11.5	
	6.0	0.8	0.80	11.0	
		0.8		11.3	

In the lower portion of Table 3 is shown the effect of pH at two different retention times for the period after the adaptation of the bacterial culture. Increasing the pH from 5.5 to 6.0 resulted in 57% (from 0.70% to 0.30%) and 27% (from 1.10% to 0.80%) reductions of residual lactose at retention times of 15.2 and 11.3 hr, respectively.

7.5 *Quality of Product from Continuous Fermentor*

The odor of each sample was checked to see if there was any indication of putrefication, and at no time was this noted.

The color of the product from the continuous fermentor was the same as that of the whey fed into the fermentor. This was in contrast to the batch fermentations, in which the color of the whey changed to a light brown as the fermentation neared completion.

The fermented product was analyzed by gas chromatography to check for the presence of products of metabolism other than lactic acid. Chromatograms of the feed and product were compared to a chromatogram of a standard solution which contained 4.4% lactic acid plus 0.1% acetic acid and 0.2% ethanol (Fig. 9). The relative amount of acetic acid and ethanol in a fermentation sample was determined by comparing the height of the respective peaks to the height of the background peak, which appeared with all samples including pure water. Peaks which appeared at the same retention time as acetic acid and ethanol were assumed to be acetic acid and ethanol peaks although a positive identification of the experimental peaks was not made. The assumption is believed to be a good one, since heterofermentative lactic acid bacteria producing CO_2 , acetate and ethanol could also propagate at the restrictive conditions within the continuous fermentor.

In Fig. 10, the upper chromatogram is from the product after 42 days of continuous operation, and the lower one is from a feed sample. These

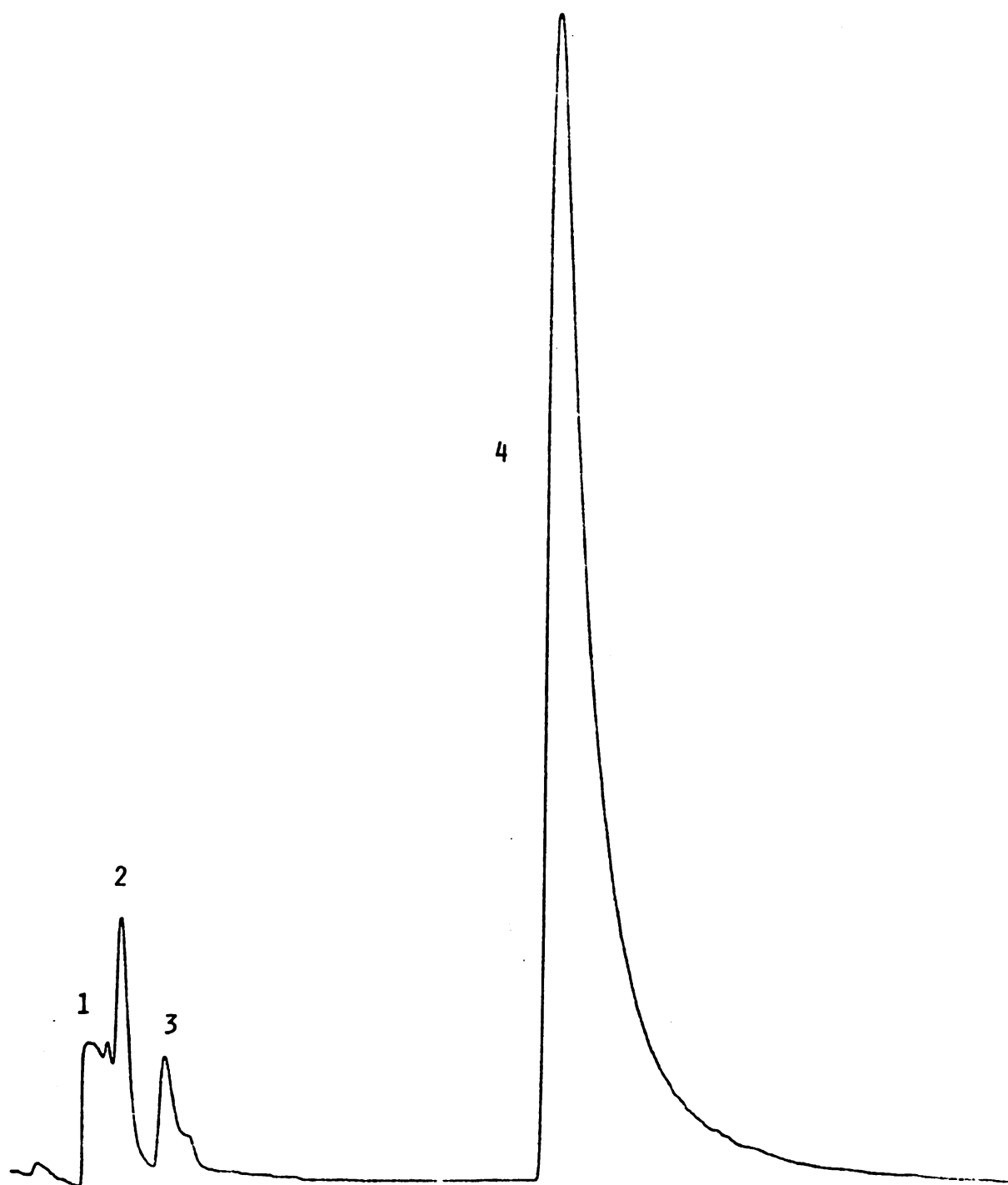


FIG. 9. Gas chromatogram of 4.4% lactic acid standard with addition of 0.2% ethanol and 0.1% acetic acid (attenuation = 16). Symbols: 1, background; 2, ethanol; 3, acetic acid; 4, lactic acid.

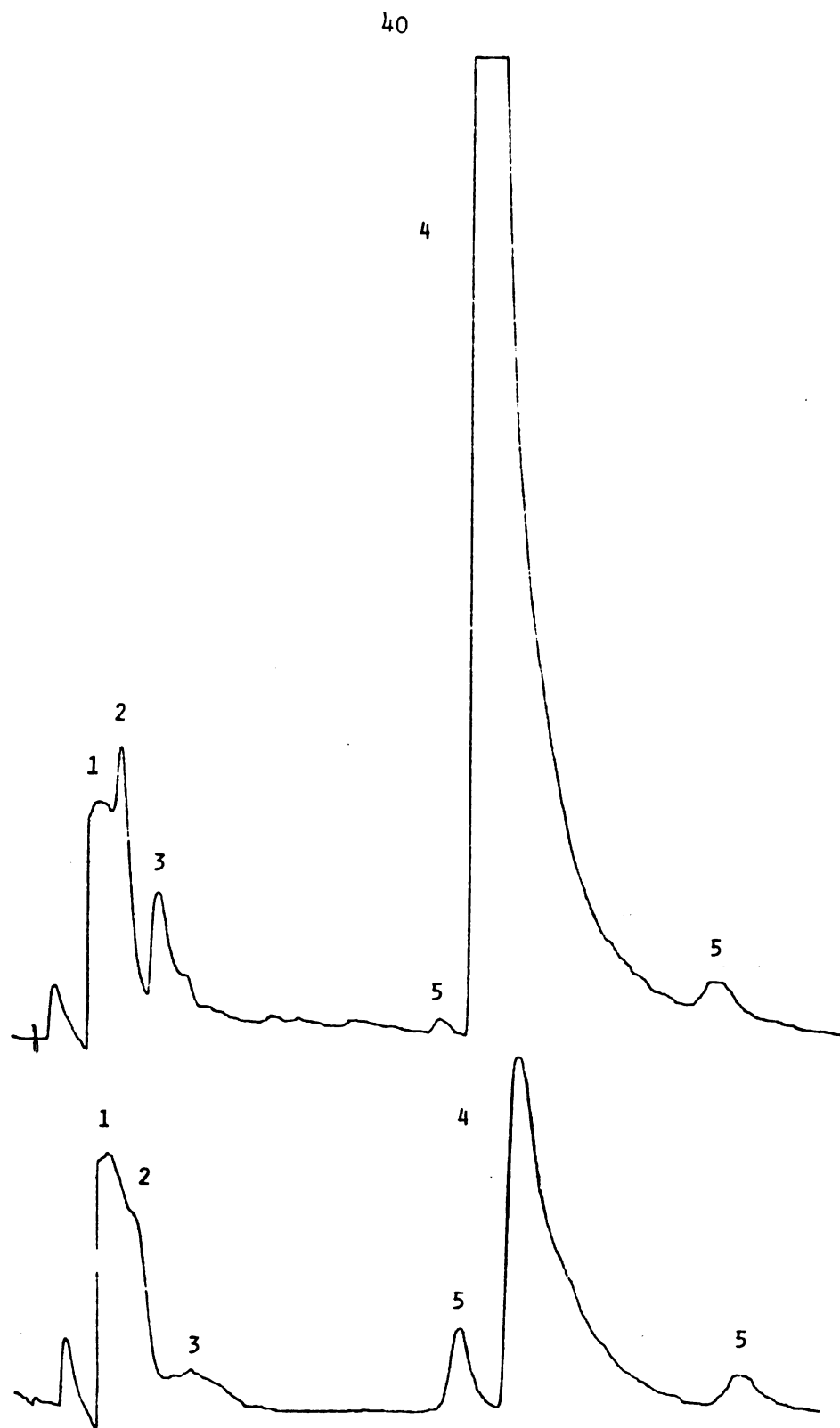


FIG. 10. Gas chromatograms of product from (top) and feed to (bottom) continuous fermentor (attenuation = 8). Symbols: 1, background; 2, ethanol; 3, acetic acid; 4, lactic acid; 5, unknown material.

chromatograms are typical of those obtained from samples taken throughout the course of the experiment. In both chromatograms, traces of ethanol and acetic acid are indicated as well as traces of two unknown compounds which elute just before and just after lactic acid. The concentrations of acetate and ethanol were greater in the product than those in the feed although they were less than the 0.1% and 0.2%, respectively, shown in Fig. 9. The concentration of the unknown compound eluting before lactic acid was substantially less in the product than in the feed, and the concentration of the unknown compound eluting after lactic acid was about the same in the product as in the feed.

Compounds that elute between acetate and lactate were not produced in the fermentation, including butyric acid which had been found to elute about one-third of the way between acetate and lactate. It was concluded, therefore, that lactic acid was in fact the predominant product of the long-term continuous fermentation and that a significant number of butyric acid-producing organisms were not propagated in the continuous fermentor.

All of the above experimental results were obtained with a continuous fermentation lasting 42 days. A preliminary continuous fermentation lasting 15 days also produced a high-quality product and essentially confirmed the above effects of pH and retention time on the concentration of residual lactose. However, mechanical problems with the feed pump and the absence of a discernible adaptation of the culture prevented a quantitative comparison of the two continuous fermentations.

7.6 *Effect of Yeast Extract and Cornsteep Liquor on Batch Fermentation*

Two series of three batch fermentations each were made to determine whether growth factors would improve the rate of fermentation in fresh whey. Two crude sources of growth factors were used: yeast extract at

levels of 0.1, 0.2 and 0.4%, and cornsteep liquor at levels of 0.25, 0.50 and 1.00%. The cornsteep liquor was autoclaved at 121 C for 30 min prior to use. The whey for all three fermentations in a series was from the same lot. Both the ammonium ion concentration and lactose concentration were used to determine the rate of fermentation.

Whey supplemented with yeast extract was fermented more rapidly and to a greater extent than unsupplemented whey. In Fig. 11 is shown the effect of this growth supplement on the accumulation of ammonia as a function of time. A typical unsupplemented fermentation is presented for comparative purposes. The unsupplemented fermentation was conducted on a separate lot of whey which contained less lactic acid at the start of the fermentation, hence the initial level of ammonia was slightly lower than in the other three fermentations. The 25 hr fermentation time was typical of the many fermentations which were conducted before it was discovered that growth factors significantly improved the rate of fermentation.

Lactic acid and residual lactose also were analyzed in samples from the series supplemented with yeast extract. The results are shown in Fig. 12 and complement those in Fig. 11. Yeast extract levels of 0.2 and 0.4% gave better results (i.e., they reduced the lactose concentration to a lower level) than 0.1% yeast extract. The higher concentrations of yeast extract reduced the fermentation time to approximately 12 hr as compared to the 24 to 30 hr encountered when no supplements were used. The residual lactose analyses for the unsupplemented fermentation were not available for comparison. However, the data for the 0.1% level of yeast extract in Fig. 11 and 12 and the level of NH_4^+ in the unsupplemented control in Fig. 11 indicated that considerable lactose (0.5 to 1.0%) remained in the product when no supplements were used.

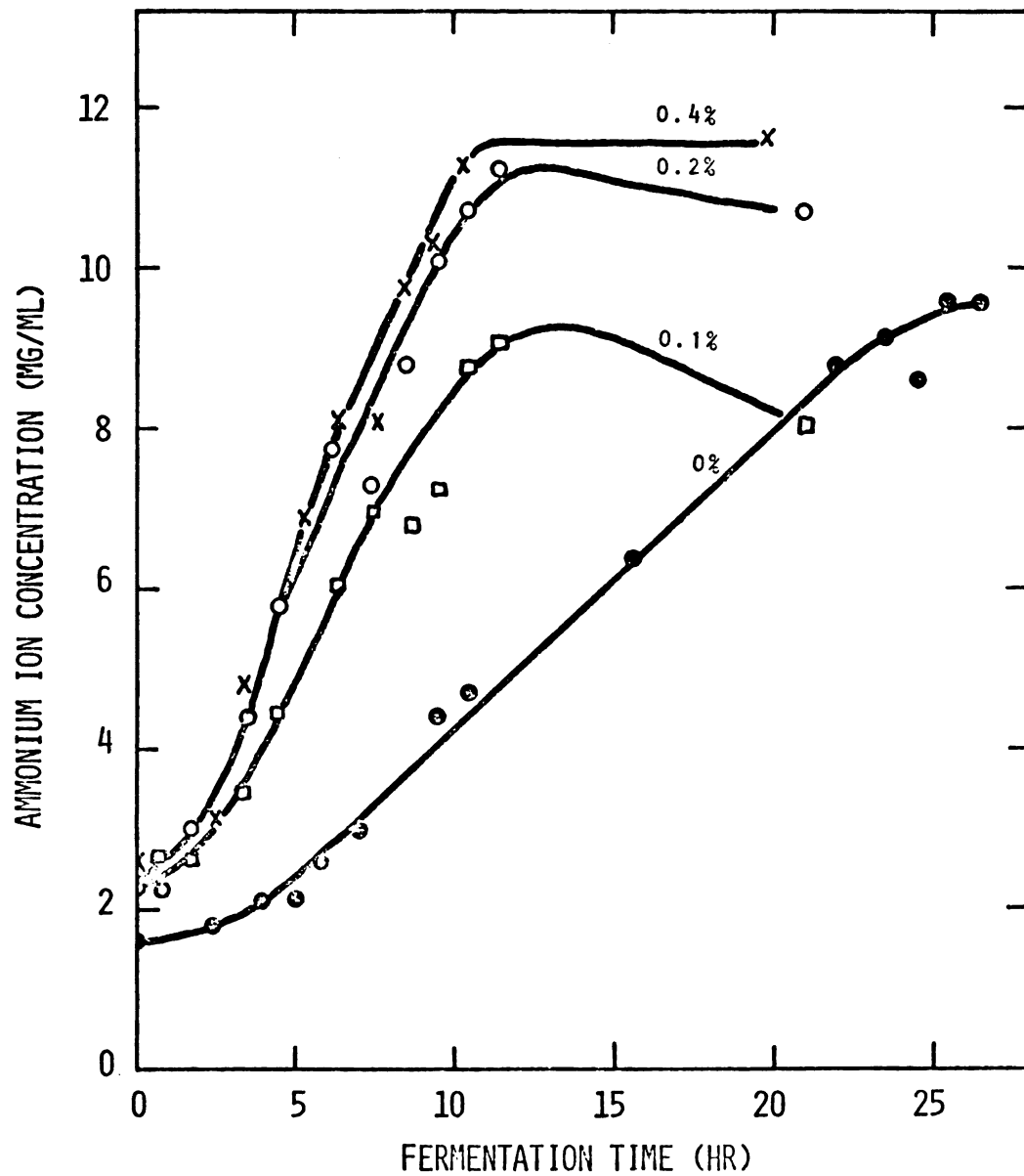


FIG. 11. Effect of three concentrations of yeast extract on the rate of batch fermentation of fresh whey, measured indirectly by ammonium ion concentration. Symbols: $\square$, 0.1%; $\circ$, 0.2%; $\times$, 0.4%; $\bullet$, typical unsupplemented fermentation.

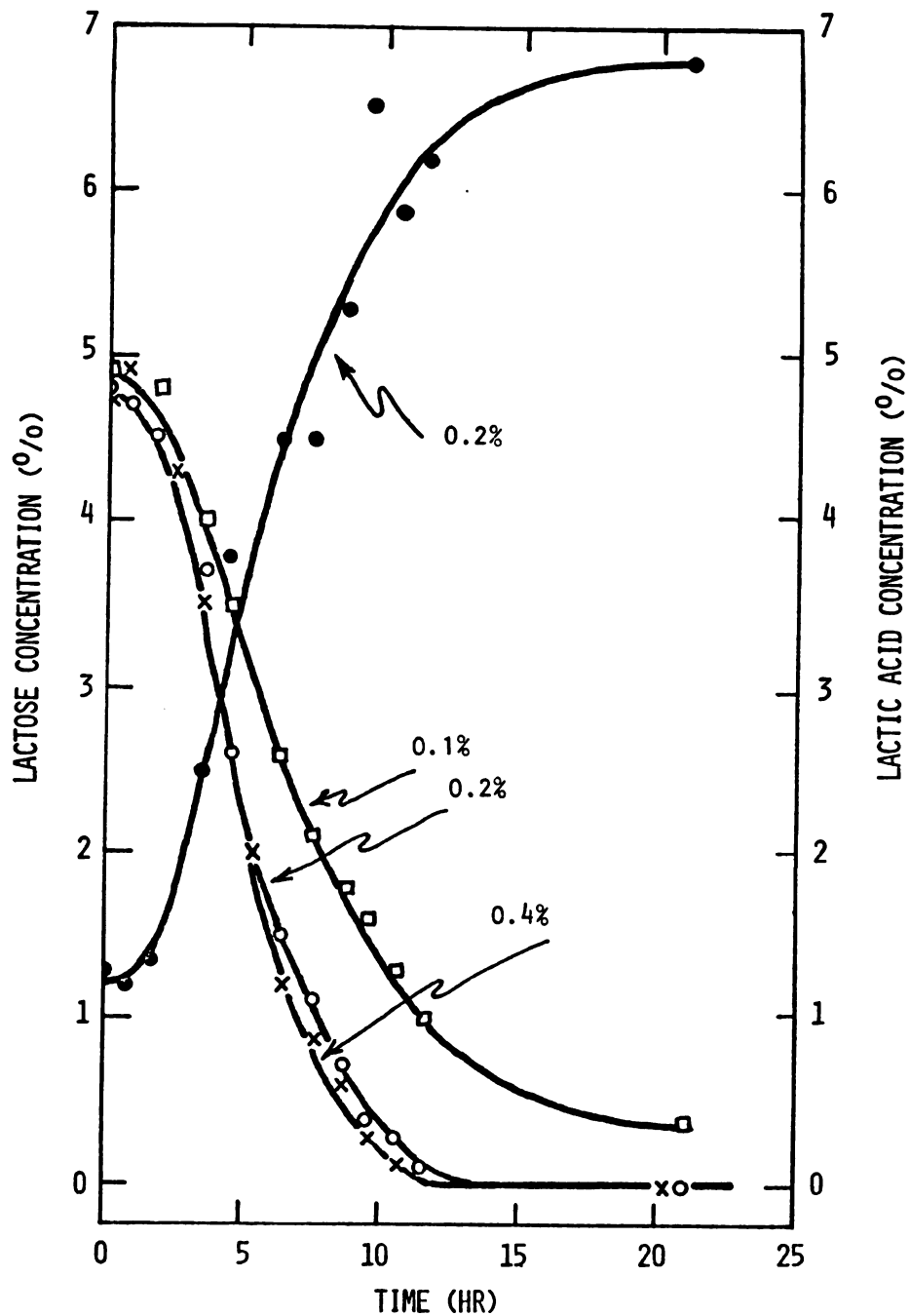


FIG. 12. Effect of three concentrations of yeast extract on the rate of batch fermentation of fresh whey as measured by the appearance of lactic acid (closed symbol) and the disappearance of lactose (open symbols).

A material balance on one of the batch fermentations indicated that no significant portion of the substrate was lost to products other than lactic acid (Table 4). Table 4 is a list of the concentrations of lactose and lactic acid at various times during the fermentation. The last column in the table is the sum of the two concentrations. The totals found in the last column are within $\pm 10\%$ of the mean. The variance is random, and the totals do not steadily decline as would happen if a detectable amount of substrate was lost to CO_2 . A second observation indicated the lack of gaseous products: the batch fermentations were conducted in a closed fermentor with a toy balloon as a safety device for releasing any excessive gas produced by the fermentations, and at no time was the balloon extended due to gas production.

In Fig. 13 is shown the effect of three levels of cornsteep liquor on the rate of fermentation. One percent cornsteep liquor gave only marginal improvement over 0.25 and 0.50% cornsteep liquor. A comparison of Fig. 12 and 13 reveals that cornsteep liquor at a level of 0.25% performed nearly as well as 0.2% yeast extract when the two were used as sources of growth factors in fresh whey.

7.7 *Ammonium Lactate and Calcium Lactate Inhibition of Batch Fermentation*

Equation 10 is based on the assumption that the specific growth rate k_i is diminished as a linear function of the product concentration. The data of Luedeking, given in Fig. 3, support but do not validate this assumption. In fact, a plot similar to Fig. 3 of k_i vs. substrate (rather than product) concentration would indicate that there also is a linear relationship between k_i and substrate concentration. To resolve whether in fact product inhibition or substrate exhaustion had the predominate effect on k_i , a series of batch fermentations were conducted in which an excess

TABLE 4. *Material balance data for a batch fermentation of fresh whey supplemented with 0.2% yeast extract*

Fermentation time (hr)	A Lactose, %	B Lactic acid, %	(A+B) Sum ^a , %
0.0	4.8	1.3	6.1
0.8	4.7	1.2	5.9
1.8	4.5	1.4	5.9
3.5	3.7	2.5	6.2
4.5	2.6	3.8	6.4
6.3	1.5	4.5	6.0
7.5	1.1	4.5	5.6
8.6	0.7	5.3	6.0
9.5	0.4	6.5	6.9
10.5	0.3	5.9	6.2
11.5	0.1	6.2	6.3
21.0	0.0	6.8	6.8

^a Mean = 6.2; standard deviation = 0.4

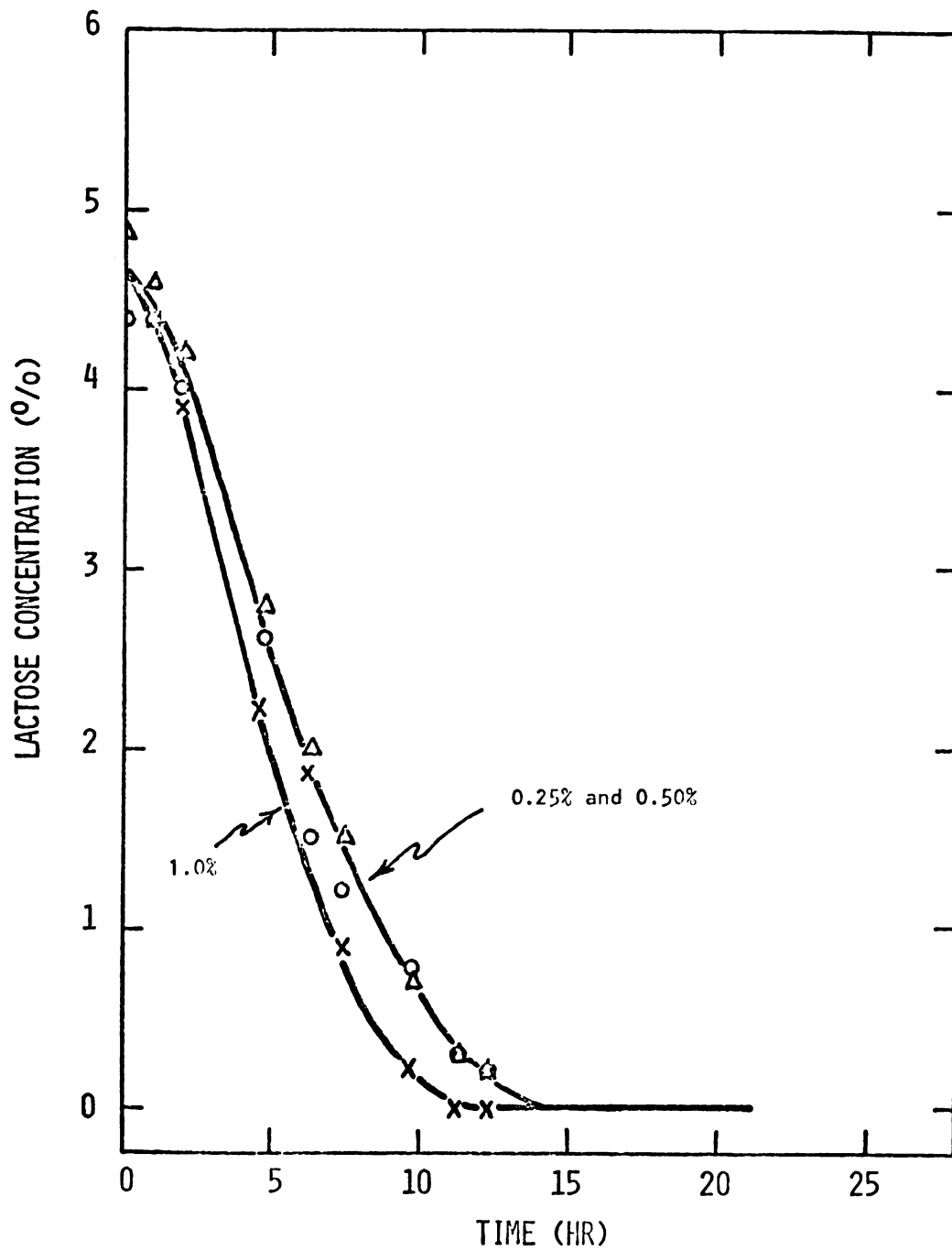


FIG. 13. *Effect of three concentrations of cornsteep liquor on the rate of batch fermentation of fresh whey. Symbols: Δ , 0.25%; $\circ$, 0.50%; $\times$, 1.0%.*

of substrate was always present and varying amounts of product were present. If each batch fermented to the same extent, it would indicate that k_i is independent of product concentration. However, if each batch fermented to an extent which was inversely proportional to the amount of product present, it would indicate that k_i is dependent on the product concentration. In the experiment, the terminal pH was used as a measure of the extent of fermentation. That is, the terminal pH indicated the extent of fermentation before k_i was reduced to 0.

Both ammonium and calcium lactate were evaluated as potential inhibitors by adding 4 ml of a bacterial culture and varying amounts of each lactate solution to 40 ml of reconstituted, unsterilized whey. The whey had been previously neutralized to pH 7.0 with 30% aqueous NH_3 .

The lactate solutions were prepared by neutralizing reagent-grade lactic acid with either ammonium hydroxide or a slurry of calcium hydroxide. The neutral calcium lactate solution contained 44% (w/v) lactate ion, and the neutral ammonium lactate solution contained 59% (w/v) lactate ion. The salt solutions were standardized at 44% (w/v) lactate ion by diluting the ammonium lactate with distilled water. The calcium lactate solution was a solid at room temperature and was liquified for transferring by heating in a boiling water bath.

The following formula was used to calculate the volume of lactate solution to be added to the whey samples:

$$\frac{40 \text{ ml whey}}{100 \text{ ml whey}} \times \frac{X \text{ g lactate}}{100 \text{ ml whey}} \times \frac{1.00 \text{ ml solution}}{0.44 \text{ g lactate}} = Y \quad (15)$$

where: X = desired percentage of lactate supplementation

Y = volume of solution to be added, ml.

Two bacterial cultures were used. One was *L. bulgaricus* #2217 which had been stored in a stab culture for five months in a refrigerator, during which time the refrigerator malfunctioned for about two weeks. Apparently, the fermentative capability of the culture changed during storage as the culture did not ferment lactose as vigorously as the *L. bulgaricus* culture used for the other parts of the experiment. The changed condition of the culture did not affect this comparative study.

The second culture was obtained from a freeze-dried sample of the insolubles recovered from the continuous fermentor on the 34th day. Both cultures were propagated in sterile, 10% skim milk. The lactate supplemented whey cultures were incubated at 42 C. After 17 and 41 hr of incubation, the pH of each sample was determined with a pH meter.

Both ammonium lactate and calcium lactate inhibited the whey fermentation, although the former was significantly more inhibitory (Fig. 14). The mixed culture was more acid tolerant than the #2217 culture. The mixed culture lowered the pH of the unsupplemented samples to about 3.9 whereas #2217 culture lowered the pH to 4.6. At all levels of supplementation the mixed culture was able to reduce the pH to values lower than those achieved by the other culture.

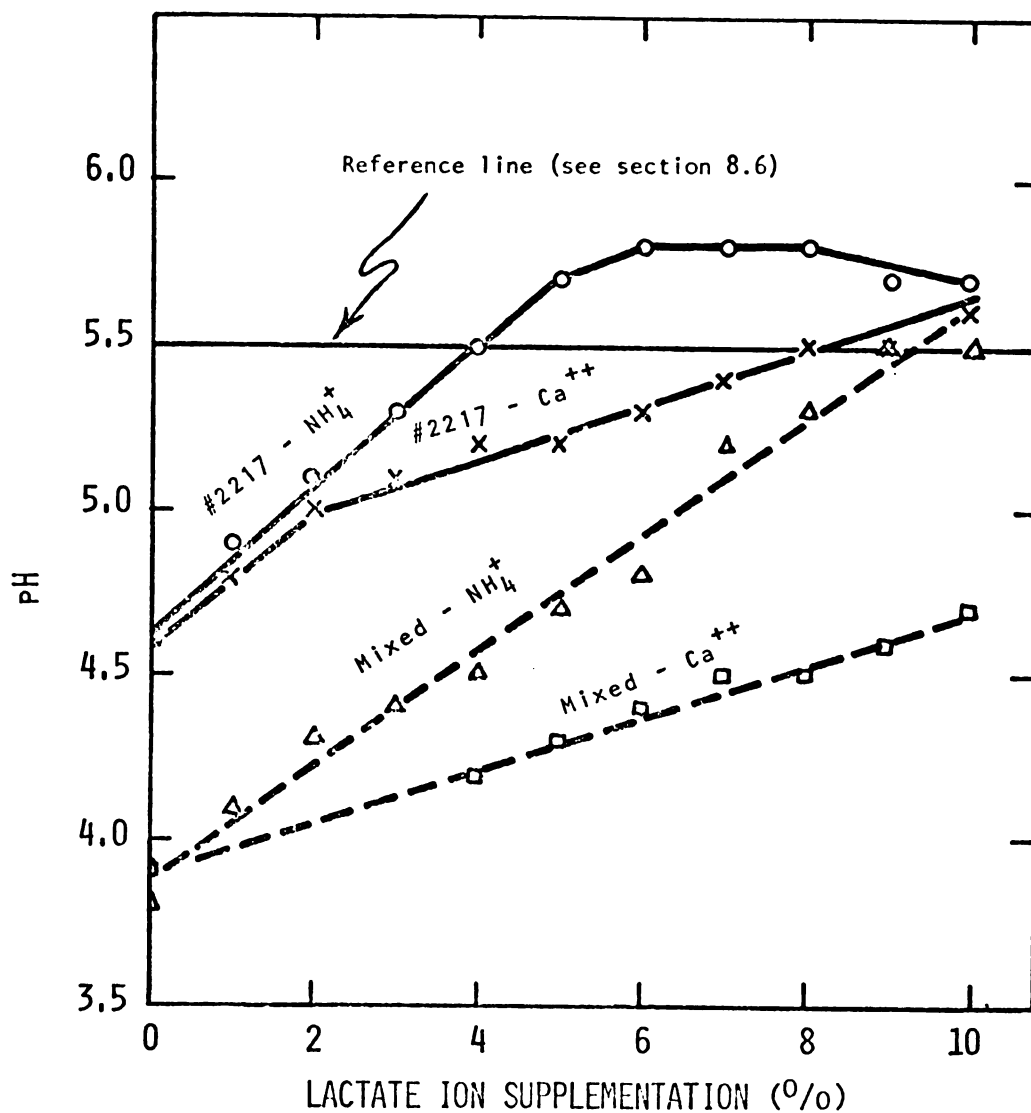


FIG. 14. Effect of ammonium and calcium lactate on the terminal pH of batch fermentations with *L. bulgaricus* #2217 or a mixed culture of lactic acid bacteria. Symbols: O, culture #2217 with ammonium lactate; X, culture #2217 with calcium lactate; Δ , mixed culture with ammonium lactate; $\square$, mixed culture with calcium lactate.

8. DISCUSSION

8.1 *Effect of Retention Time and Staging on Conversion of Lactose*

A comparison of the results from the batch fermentation model and the experimental data (Fig. 4) shows how well the model fits the data. The model, therefore, is a good simulation of the *batch* data.

Care, however, must be exercised in applying the batch model to a continuous system (20,62). Due to product inhibition, many of the kinetic advantages of continuous fermentation are not fully realized in the lactic acid fermentation. Approximately the same time was required to process a given volume in the continuous fermentor as in the batch fermentor. However, the use of staging and increasing the pH would reduce the required retention time in the continuous fermentor. In spite of the fact that the retention times in a batch and continuous fermentor may be approximately the same, continuous fermentation would likely be the preferable mode of operation since it is more adaptable to instrumental control, is better integrated into the preceding and subsequent processing operations, and generally yields a more uniform product.

Equation 12 predicts that the bacterial population will increase as long as the throughput rate $r < k_i S / (K_s + S)$. In a fermentation in which the specific growth rate k_i is a constant, the population will increase until the substrate S is nearly exhausted. Therefore, the fermentation always operates near the point of substrate exhaustion. If $r > k_i S / (K_s + S)$, the bacterial culture is washed out of the fermentor.

In the lactic acid fermentation, however, k_i is not a constant but rather varies as some function of product concentration (Fig. 7). As r

is decreased (i.e., retention time is increased), the product concentration increases and causes k_i to decrease. Therefore, further increases in retention time are counteracted by a reduction in the specific growth rate. Hence, the curve in Fig. 5 flattens, and very little improvement in lactose conversion is achieved by increasing the retention time beyond the optimum.

The second stage of a two-stage fermentation does not depend on bacterial growth alone for maintaining the population within the second fermentor. The second stage receives a supply of bacteria from the first stage. Therefore even if k_i is zero for the second stage, there will be bacteria in that stage as long as $r < k_i S / (K_s + S)$ in the first stage. The bacteria that are fed to the second stage can use maintenance metabolism to dissimilate lactose even if $k_i = 0$. This fact, along with a low concentration of lactose in the feed to the second stage, account for its effectiveness.

It should be pointed out that k_i has been presented as a function of product concentration and not of substrate concentration. It appears that a fermentation with an initial substrate concentration of less than 5% would benefit relatively little by staging (i.e., the curves in Fig. 6 would be shifted downward). Conversely, a fermentation with an initial substrate concentration in excess of 5% would receive relatively more benefit from a multi-stage fermentor than demonstrated in Fig. 6.

From a practical standpoint, this would indicate that cheddar cheese whey (4.9% lactose, 0.2% lactic acid) may do adequately in a single-stage fermentor, while cottage cheese whey (5.8% lactose, 0.7% lactic acid) may benefit from staging. Whey fortified with additional solids from condensed whey may also benefit significantly by staging. The addition of molasses or other concentrated substrate to whey to increase the sugar

concentration may also benefit by a multi-stage fermentor. The purpose of adding the concentrated substrates to whey would be to reduce the amount of water that must be removed to get a pound of concentrated product. For example, the addition of enough molasses to whey to double the substrate concentration would cut in half the amount of water that must be removed to get a pound of product.

Once a valid mathematical model of the lactic acid fermentation is available, it can be used to evaluate not only various substrate concentrations (as outlined above) but also various fermentor designs. Plug-flow fermentors with a portion of the product used as a continuous inoculum (24), multi-stage fermentors with stages of varying sizes, dialysis fermentors (57) and ultrafiltration fermentors (62) are a few systems which could be evaluated with the simulation. In each case experimental results must be used to verify the model and to determine if the culture shifts to a different metabolic pathway (62).

In this study, the model aided in the interpretation of the limited data from the two-stage fermentor and predicted the performance of a three-stage fermentor (Fig. 8). The experimental data indicate that a lactose conversion of more than 98% can be achieved with a retention time of 15.5 hr in each of two stages. The model predicts that it may be possible to achieve the same conversion with a retention time of 11 hr in each of two stages or 5.5 hr in each of three stages.

To fit the model to the experimental data it was necessary to find the relative values of the proportionality constants α and β . It should be noted that the relative values of α and β (i.e., the ratio of α to β) and not the absolute values were critical to fitting the model to the experimental data. To demonstrate that other values of α and β could

also force the model to fit the data, values of 2α and 2β were substituted for α and β . The resulting product and substrate concentrations remained the same as when α and β were used in the program; however, the apparent bacterial density was reduced to one-half the previous value.

The true values of α and β could be found if the true bacterial density were known at any *one* retention time. For the purposes of this study the relative values of α and β served just as well as the true values, since the bacterial density was of no particular interest. Another reason for ignoring bacterial density was the difficulty in obtaining a value for it in a turbid medium such as whey. Plate counts are of little value due to the tendency of lactobacilli to form chains. DNA composition of the fermented medium may be an indirect method of determining bacterial numbers since each cell contains a fixed amount of DNA (17).

The relative values of α and β can be used to determine the relative contributions of bacterial growth and maintenance metabolism to lactic acid production. For example, in a single-stage whey fermentation with a retention time of 5 hr, the residual lactose concentration is 4.2% (Fig. 8); the product concentration is 1.6% (1.0% in feed + 0.6% produced in the fermentor); and k_i equals 0.2 (Fig. 7). Since αk_i accounts for product formation due to bacterial growth and β accounts for product formation due to maintenance metabolism, the respective contributions are 0.44 and 0.20 when $\alpha = 2.2$ and $\beta = 0.2$. Therefore, in this situation 70% of the lactic acid is formed due to bacterial growth, and 30% is formed due to maintenance metabolism. In the same fermentor with a retention time of 30 hr (i.e., $k_i = 0.03$), $\alpha k_i = 0.07$ and $\beta = 0.2$.

Therefore in this situation, only 25% of the lactic acid is formed due to growth, and 75% is formed due to maintenance metabolism.

8.2 *Adaptation of the Continuous Culture*

The data in Tables 2 and 3 indicate that a definite change took place in the culture during the 42 days of continuous fermentation. This change has been loosely referred to as an "adaptation". It was never elucidated, however, whether in fact this was the selection of a mutant, the replacement of culture #2217 by another lactic acid bacterial species or a symbiotic association of #2217 with a mycoderm or other bacterial species. Any one or a combination of the above changes could have taken place in the culture.

Whatever the actual change, it was for the overall benefit rather than detriment of the whey fermentation. After the adaptation, it was possible to increase the throughput rate by a factor of 3 and still achieve the same degree of conversion of lactose that was achieved at the lower rate before adaptation. For example, before the adaptation a retention time of 22 hr and a pH of 5.5 resulted in a residual lactose level of 2.1%. After the adaptation a retention time of 7.6 hr at the same pH resulted in a residual lactose level of 2.3%.

The adaptation period appears to have been a discrete time period. After that period the culture was stable, and the results were reproducible. It is possible that prolonged operation of the continuous fermentor would result in the selection of a third culture which was even more efficient than the culture which was present at the time the fermentation was terminated.

The adaptation of the bacterial culture supports the argument that a continuous fermentor must be operated for an extended period of time

before much significance can be applied to the results. It has been arbitrarily stated that a continuous operating time of 1000 hr should be achieved before a fermentation system is called "continuous" (61). This is proposed because so many changes can take place in a continuous fermentor, and only time will determine if those changes will in fact affect the system in question.

The use of unsterilized medium and equipment make it very easy for contaminants to enter the fermentor. Any time a faster growing species enters, it will displace the predominate species. However, the restrictive conditions in the fermentor (i.e., low pH, high temperature, anaerobic conditions, lactose substrate and possible antibiotics produced by the lactobacilli) make it very unlikely that species other than lactic acid bacteria will predominate.

To produce a cattle feed supplement from whey, it makes no difference what the species of bacteria is as long as it produces predominately lactic acid and is non-pathogenic for cattle. It is extremely unlikely that any pathogenic species could survive the restrictive conditions imposed on the whey fermentation. This conclusion is drawn from the fact that lactic acid fermentations are used as a "natural" means of preservation for such items as cheese, pickles, sauerkraut, and silage (63). The occurrence of pathogenic bacteria is not a problem when the above items have been processed properly.

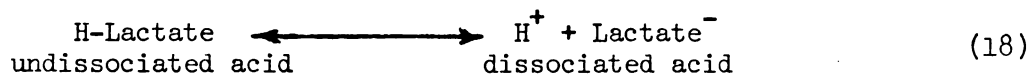
8.3 *Effect of pH in the Range of 5.5 to 6.0 on Continuous Fermentation*

The effect of pH on the continuous fermentations of this study confirms that the ammonium lactate system is affected by pH in a manner similar to the calcium lactate (37) and sodium lactate (20) systems. The

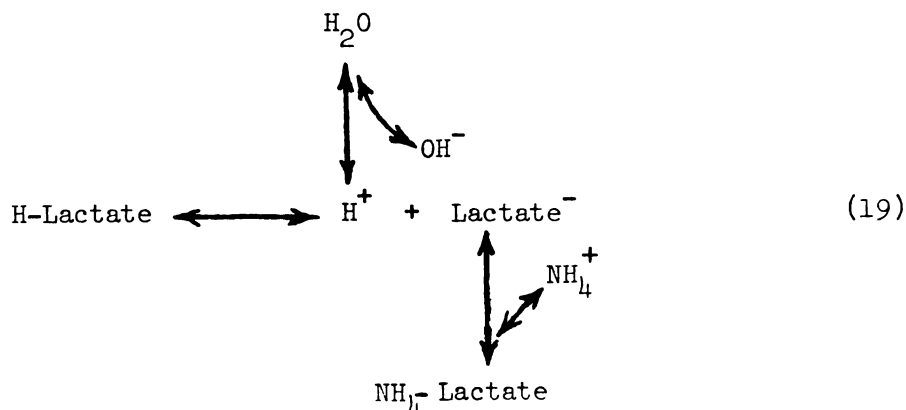
data indicate that, for a given flow rate, more lactose is converted at pH 6.0 than at pH 5.5. A logical conclusion would be to operate the system at pH 6.0. However, other species can also grow more efficiently at the higher pH. During most of these studies the pH was kept at 5.5 to discourage contamination while allowing the lactobacilli to ferment at a reasonable rate.

The pH setting of 5.5 was selected because it was known that relatively few organisms tolerate this low pH at a temperature of 44 C. However, some clostridia can ferment lactic acid to butyric acid even at these restrictive conditions of pH and temperature (9, 37). There must be another factor which accounts for the fact that no butyric acid or spore-forming bacteria were observed in the product.

To better understand the "other factor" which prevents contamination of the continuous whey fermentation, recall that a weak acid in an aqueous medium is only partially dissociated. For example,



Also recall that the presence of a salt of the lactate influences the concentration of lactate ions in the system; and, in turn, the concentration of undissociated acid:



For a given pH (i.e., for a given concentration of H-ions), any addition of ammonium lactate to the system must result in an increase in the concentration of undissociated lactic acid. How much of the lactate from the added salt is converted to undissociated lactic acid will depend on the degree of dissociation of the salt at the given pH and temperature.

It was demonstrated in 1928 that the concentration of undissociated lactic acid is more directly related to the inhibition of lactic acid bacteria than the actual pH of the medium (55). More recently, a generalized model for the effect of pH on the biological activities of weak acids and bases was developed by Simon and Beevers (59). Both studies indicate that it is mainly the concentration of undissociated acid that inhibits the activity of a bacterial culture. For a given medium, there is a maximum concentration of undissociated lactic acid which a bacterial species can tolerate.

The high concentration of undissociated lactic acid, therefore, is very likely the "other factor" which makes it possible to operate the continuous whey fermentation at a pH above what would normally be considered necessary to exclude butyric acid-producing bacteria.

Because of the high concentration of undissociated lactic acid in the product, it is quite likely that the continuous fermentation could be operated safely at a pH above 5.5 and thereby take advantage of the higher fermentation rates obtainable at the higher pH. During this study the process was operated at pH levels up to 6.0 without noticeable contamination. However, the process was not operated at the higher pH long enough to conclude definitely that no contamination would result. Because of the significantly better conversion of lactose to lactic acid at the higher pH levels, a very productive project would be the determination of the

maximum pH which will still exclude undesirable microorganisms.

The above discussion also leads to the conclusion that particular attention must be paid to pH when operating a multi-stage continuous whey fermentation. The concentration of ammonium lactate and hence the concentration of undissociated lactic acid would be lower in the first stage than in the final stage. Therefore, a lower pH must be maintained in the first stage to maintain the same degree of inhibition obtained by a higher pH in the final stage.

The above discussion can also be related to batch fermentations. At the time of inoculation of a batch culture, the product concentration is low. Therefore, a relatively low pH would be required to obtain a given degree of inhibition of contaminants, and a pH of 5.5 has proven satisfactory for this purpose. The general practice has been to maintain the initial pH throughout the entire course of the fermentation. However, it should be possible to program the pH so that it is raised as the fermentation progresses and still maintains adequate inhibition of contaminants. By raising the pH as the fermentation progresses, the product would be less inhibitory to the lactic acid bacteria while still inhibiting the less acid tolerant species. The net effect should be a significant reduction in batch times.

A recent publication by Hanson and Tsao (20) states that lactate concentrations in the range of 0 to 2% were not inhibitory to the lactic acid bacteria used in that study. The reduction in the specific growth rate for the bacteria during the progress of the fermentation was attributed to the exhaustion of substrate. In view of the above discussion on product inhibition and in view of a study which used dialysis culture to prove product inhibition (16), it is difficult to accept the view that a

substrate concentration in excess of 10 g per liter was limiting the rate of fermentation. The fact that product inhibits lactic fermentations accounts for the extremely large values ($> 20,000$ mg per liter) reported for the saturation constant K_s in the Monod equation.

The value of K_s is actually very low. For bacteria growing in carbohydrate substrates, it lies in the order of magnitude of decades of milligrams per liter of medium (11). Lactic acid fermentations are normally considered complete when the substrate level has been reduced to less than 1,000 mg per liter (56). Assuming a value of 10 mg per liter for K_s , it can be demonstrated that the term $S/(K_s + S)$ is of little consequence even at the "end" of a lactic acid fermentation.

$$\frac{S}{K_s + S} = \frac{1,000}{10 + 1,000} = \frac{1,000}{1,010} = 0.990 \approx 1 \quad (20)$$

Hence, K_s in the study by Hanson and Tsao was more likely an indication of product inhibition rather than substrate exhaustion. Along with the fact that maintenance metabolism was ignored, this may explain why their mathematical model was not accurate when it was applied to continuous culture.

Finally, the concept of the interaction between the neutralized lactic acid and undissociated lactic acid helps to explain the difference between the inhibitory effect of ammonium lactate and calcium lactate. The amount of undissociated acid produced by the addition of the salt depends on the degree of dissociation of the salt. Ammonium lactate, which is a salt of weak acid and a weak base, will dissociate to a higher degree than calcium lactate, which is a salt of a weaker base. Therefore, it should be necessary to add more calcium lactate than ammonium lactate to whey to obtain total inhibition of a bacterial species at a given pH.

In fact this happens. With reference to the horizontal line at pH 5.5 in Fig. 14, it took 4% lactate as ammonium lactate to inhibit culture #2217 at pH 5.5. At the same time it took 8% lactate as calcium lactate to inhibit culture #2217 at the same pH. If the concentration of undissociated lactic acid were determined, it should be about the same in both cultures if the above argument holds.

8.4 *Quality of Product from Continuous Fermentor*

The gas chromatograph is a very useful tool for evaluating the quality of fermentation products. Besides an approximate analysis for lactic acid, it also analyzes for other short-chain metabolic products. The presence of other metabolic products besides lactic acid would be positive indication of contaminating microorganisms (19). Fortunately, no products other than lactic acid plus traces of ethanol and acetate were found in the product from either the batch or continuous fermentor. Therefore, it is concluded that the lactic acid bacteria were not replaced by another group of bacteria even after operating the continuous fermentor non-aseptically for 42 days.

Not only is it important that lactic acid is the sole product of fermentation, but it is also important that the product contain as low a concentration of lactose as practical. Residual lactose is not desirable in the final product due to the fact that it crystallizes after the product is condensed. The lactose crystals precipitate in the product storage tanks to give a troublesome sludge, and they plug the product transfer lines. A reasonable goal would be a residual lactose concentration of less than 0.1% in the final product. This is easily achieved in vigorous batch fermentations, and it can be achieved in a continuous fermentation if more than one stage is used.

8.5 *Effect of Yeast Extract and Cornsteep Liquor on Batch Fermentation*

The beneficial effect derived from yeast extract and cornsteep liquor in the batch fermentations might seem surprising in view of the fact that milk is often referred to as "nature's most perfect food". Whey is simply milk from which some protein and the butterfat have been extracted. Very few bacteria utilize lipids (e.g., butterfat) as nutrient sources, and whey retains a significant amount of protein (0.9%). Therefore, whey might be thought of as a "nearly perfect food" for bacteria.

However, lactic acid bacteria are known to be extremely fastidious organisms (47, 60, 64). It is not unusual for lactic acid bacteria to be stimulated by various sources of growth factors even when the lactic acid bacteria are growing in complex media such as whey (7, 28, 53, 73). The results bore out this latter information.

In a commercial operation to produce a high-protein cattle feed supplement from whey, it may be beneficial to supplement the whey with a source of growth factors such as cornsteep liquor. Arnott (3) recommended the use of a 10% inoculum to give a fermentation time of about 14 hr and to prevent spoilage. In approximately 50 pilot-scale batch fermentations conducted at Michigan State University (23) concurrently with this study, 2.5 to 5.0% inoculum was used along with 0.7% cornsteep liquor; and the fermentations were completed in approximately 14 hr. The use of 3 gal of sterile cornsteep liquor made it possible to save 25 to 38 gal of culture medium (skim milk). The cornsteep liquor cost approximately \$1.20 and resulted in a savings of approximately \$15.00 worth of skim milk. Additional cost savings could be expected from the ease of storing the stable cornsteep liquor rather than the perishable milk. The cost-saving benefits from the use of growth factors would be less, however,

if whey were used as the inoculum medium or if a portion of a previous fermentation were used to inoculate a batch of whey.

Although growth factors were not used in the present continuous fermentation studies, it is conceivable that the retention time required to achieve 98% lactose conversion could be reduced by the use of growth factors. Growth factors may be particularly beneficial in a multi-stage fermentation in which the first stages are not operating at high product concentration. Moreover, it has been demonstrated that lactobacilli are more acid tolerant in enriched media, and this phenomenon would likely benefit a supplemented continuous fermentation (55).

In addition to yeast extract and cornsteep liquor, there are many other sources of growth factors which might prove equally useful in lactic acid fermentations. Other natural plant extracts have been used to stimulate fermentations: malt sprouts (7) and alfalfa solubles (33, 52) are two examples. A common practice is to use a mixed culture of a myco-derm to supply growth factors for the bacteria (73). In some instances, Maillard reaction products (28) and formic acid (66) stimulate lactic acid bacteria. Various pure compounds (e.g., riboflavin, pantothenic acid, nicotinic acid and biotin) are periodically used to supplement lactate fermentations (53, 60). Therefore, a wide variety of materials are available for evaluation as sources of growth factors in whey fermentation.

8.6 *Ammonium Lactate and Calcium Lactate Inhibition of Batch Fermentation*

The simple test used to study the effect of ammonium and calcium lactate on the fermentation of whey yields a considerable amount of information. First, the acid tolerance of a given strain of lactic acid bacteria can be evaluated with the test. This is easily done by inoculating

unsupplemented samples of whey with the culture in question and checking the final pH after 30 to 40 hr of incubation. This test is useful for evaluating the acid tolerance of a strain, but it tells nothing about the *rate* at which the strain converts the substrate to lactic acid.

Some lactic acid bacteria ferment the substrate very rapidly as long as the concentration of product is quite low, but the fermentation rate is considerably reduced as the product concentration increases. *Streptococcus lactis* is an example of a fast-fermenting but acid-sensitive species. Other species ferment the substrate more slowly but are less sensitive to the acid product (e.g., *Lactobacillus bulgaricus*). Similar variations occur in strains within a species.

Generally it is desirable to have an acid tolerant species for continuous fermentation because of the continually high concentration of product. For batch fermentations the acid tolerance of the species is not as critical if a relatively low concentration of sugar is fermented. As the initial concentration of sugar is increased, the ability of the culture to tolerate the product becomes more important.

The *relative* acid tolerance of the two cultures which were tested is independent of the amount of product present. In Fig. 14 the two curves representing calcium lactate inhibition are nearly equidistant, and the same generally holds true for the two curves representing ammonium lactate inhibition. The top ammonium lactate line very likely plateaus because the initial pH was not high enough to permit bacterial growth (see Tables 5 - 8 in Appendix). The pH in the plateau region was reduced slightly from the initial value. This may have been the result of maintenance metabolism before death of the culture.

The approximate amount of substrate which can be fermented by a

species under conditions of controlled pH can be estimated from Fig. 14. This estimation can be made by drawing a horizontal line across the graph at the level at which the pH is to be controlled. At the point of intersection between the horizontal line and the product inhibition curve, one can read the maximum amount of lactate which the culture can tolerate at that pH. For example, at pH 5.5 Culture #2217 could be expected to tolerate a maximum lactate ion concentration of approximately 4% when ammonia was the neutralizing agent and approximately 8% when calcium hydroxide was the neutralizing agent. These values are only approximations because some additional ammonium lactate (approximately 1%) was present in all the samples as a result of neutralizing the whey with ammonium hydroxide and because no accounting was made of the increase in volume caused by the addition of the lactate solutions. A more precise estimate of the maximum amount of fermentable substrate could be made by reconstructing Fig. 14 after analyzing each culture for the amount of lactate present. This analysis was not done since the original experiment was not designed to estimate the maximum fermentable substrate and because the difference between the estimate given and the more precise value is not expected to be very great.

The above example indicates that, for a lactic fermentation neutralized with ammonia, the maximum amount of fermentable sugar is 4 to 5% which is somewhat lower than previous experience (5 to 7%). The difference is likely due to the fact that the culture used in this particular experiment was inadvertently changed due to prolonged storage. In an earlier test with a culture of #2217 which had been maintained by repeated transfers to fresh medium, the culture was able to tolerate a pH of 3.8 in an unsupplemented culture.

Finally, Fig. 14 illustrates that a given culture of lactic acid bacteria can ferment much more sugar if the acid product is neutralized with calcium hydroxide rather than ammonium hydroxide. This information is important to bear in mind when attempting to relate commercial lactic acid production (in which neutralization is accomplished with calcium hydroxide or carbonate) to the production of a cattle feed supplement from whey. The cattle feed serves as a source of nitrogen for the cattle. Therefore, ammonia is the neutralizing agent of choice. However, the use of ammonia to maintain a pH of 5.5 restricts the maximum level of fermentable substrate to a level (5 to 7%) considerably below the 10 to 20% used in commercial lactic acid fermentations.

9. APPENDIX

TABLE 5. *Values for pH obtained during batch fermentations of whey supplemented with various levels of calcium lactate and inoculated with L. bulgaricus #2217*

Lactate ion, %	pH after			
	lactate addition	inoculation	17 hr	41 hr
0	6.7	6.5	4.7	4.6
1	6.4	6.2	5.1	4.8
2	6.3	6.1	5.3	5.0
3	6.2	6.0	5.4	5.1
4	6.2	6.1	5.5	5.2
5	6.2	6.1	5.5	5.2
6	6.1	6.1	5.6	5.3
7	6.2	6.0	5.6	5.4
8	6.2	6.1	5.7 ^a	5.5 ^a
9	6.2	6.0	5.7 ^a	5.5 ^a
10	6.2	6.1	-	-

^a Very viscous slurry

TABLE 6. *Values for pH obtained during batch fermentations of whey supplemented with various levels of calcium lactate and inoculated with a mixed culture of lactic acid bacteria*

Lactate ion, %	pH after			
	lactate addition	inoculation	17 hr	41 hr
0	6.6	6.3	4.4	3.9
1	-	-	-	-
2	-	-	-	-
3	-	-	-	-
4	6.1	6.0	4.9	4.2
5	6.1	6.0	5.0	4.3
6	6.1	5.9	5.1	4.4
7	6.1	6.0	5.1	4.5
8	6.1	6.0	5.1	4.5
9	6.2	6.0	5.1 ^a	4.6 ^a
10	6.2	5.9	5.1 ^a	4.7 ^a

^a Very viscous slurry

TABLE 7. *Values for pH obtained during batch fermentations of whey supplemented with various levels of ammonium lactate and inoculated with a mixed culture of lactic acid bacteria*

Lactate ion, %	pH after			
	lactate addition	inoculation	17 hr	41 hr
0	6.7	6.4	4.2	3.8
1	6.8	6.5	4.5	4.1
2	6.9	6.4	4.8	4.3
3	6.9	6.4	4.9	4.4
4	6.7	6.3	5.2	4.5
5	6.6	6.2	5.4	4.7
6	6.5	6.1	5.6	4.8
7	6.4	6.1	5.7	5.2
8	6.3	6.0	5.8	5.4
9	6.2	5.9	5.6	5.5
10	6.2	5.9	5.7	5.5

TABLE 8. *Values for pH obtained during batch fermentations of whey supplemented with various levels of ammonium lactate and inoculated with L. bulgaricus #2217*

Lactate ion, %	pH after			
	lactate addition	inoculation	17 hr	41 hr
0	6.6	6.6	4.7	4.6
1	6.6	6.7	5.1	4.9
2	6.7	6.7	5.3	5.1
3	6.6	6.6	5.6	5.3
4	6.6	6.6	5.8	5.5
5	6.5	6.5	5.9	5.7
6	6.4	6.3	6.0	5.8
7	6.2	6.3	6.0	5.8
8	6.2	6.2	6.0	5.8
9	6.1	6.1	5.9	5.7
10	6.1	6.1	5.9	5.7

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