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STERILIZATION OF NUTRILITES
ON THE RATE AND YIELD OF LACTIC
ACID FERMENTATION

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A COMPARISON OF THE EFFECTS OF GAMMA RADIATION AND HEAT
STERILIZATION OF NUTRILITES ON THE RATE AND YIELD OF LACTIC ACID
FERMENTATION

by

Robert Arthur Gillies

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The preparation of sterile media containing the fermentable substrate and nutrilites in an essentially undamaged condition is highly important in biochemical processes. At the present time, most fermentation mashies are sterilized with steam. This is unfortunate because heat may damage some of the nutrilites needed by the organisms employed. Furthermore, there are industrial and laboratory mashies that cannot be sterilized with heat because essential enzymes present in the raw materials would be inactivated.

It is well known that ionizing radiations will sterilize many materials. Consequently, gamma radiation could serve as an alternative for heat in the preparation of sterile fermentation mashies. Therefore, the present investigation was carried out to compare the effects of heat and radiation sterilization of malt sprouts and corn steep liquor on the subsequent rate and yield of lactic acid formation during the fermentation of glucose by Lactobacillus delbrueckii.

Gamma rays from cobalt-60 were used as the source of radiation. Complete sterilization was obtained with 2.5 million rep which was the lowest dosage used. However, the study included dosages as high as 4.5 million rep, in order to observe the effects of increased amounts of radiation on the nutrilites.

All fermentations were carried out under aseptic conditions, and continuous automatic pH regulation was employed. The instantaneous rates of acid production were calculated from the slope of a curve made by an instrument that continuously recorded the amount of neutralizing solution added. The rates of acid production were converted into lactic acid values after the mashies were chemically analyzed.

Two parallel series of fermentations were carried out with each nutrilitie source. In one series, the basal ingredients and nutrilities were sterilized by heat; in the other, the nutrilities were sterilized independently with gamma radiation from cobalt-60 and were then added aseptically to the fermentation medium immediately before inoculation. For controls, several fermentations were carried out using unsterilized nutrilities and also using mashies in which all the components were sterilized in their final form with radiation. The maximum rates of acid production and the yields of lactic acid obtained under the above conditions were compared.

Three general observations were made in this study. (1) The maximum rates of lactic acid formation increased from forty to seventy per cent when radiation sterilized malt sprouts were substituted for heat sterilized malt sprouts in the mashies. For example, in a typical pair of fermentations an increase in the maximum rate of lactic acid formation from 0.313 to 0.542 grams per 100 milliliters of mash per hour occurred. The yields of lactic acid varied from 79.2 to 89.1 per cent regardless of the method of sterilization. (2) The maximum rates of lactic acid production decreased from thirteen to twenty per cent while the yields were reduced by six to eleven per cent when radiation was used in place of heat for sterilizing the corn steep liquor. A decrease

in the maximum rate of lactic acid formation from 0.488 to 0.360 grams per 100 milliliters of mash per hour occurred in one pair of fermentations and is typical of the results obtained with corn steep liquor. A decrease in the yield of lactic acid from 93 to 84.6 per cent was observed in the same pair of fermentations. (3) It was found more practical to sterilize the nutrilit sources independently in the concentrated form rather than as diluted components of the fermentation mashes.

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INTRODUCTION

Radiation sterilization presently is being investigated for several industrial applications and has been suggested for use in the preparation of fermentation mash (1). However, since no experimental data has been reported for this application and, also, since atomic fission products may soon become available for industrial use, it was considered desirable to investigate the characteristics of fermentations in which the nutrilites had been sterilized with gamma radiation. Nutrilites have been defined by Williams (2) to include all organic substances, regardless of their nature, which in minute amounts are of importance in the nutrition of microorganisms.

At the present time, fermentation mash is sterilized with steam and no reliable alternates are available. This is unfortunate because heat may damage some of the medium constituents. For example, Pan, Peterson and Johnson (3) have shown that the yield and rate of lactic acid production by fermentation varies inversely with the severity of the heat treatment used in preparing the medium.

Ionizing radiations offer many potential advantages. For example, gamma rays have great penetrating power, they do not induce radioactivity in the sterilized product and, since there is no appreciable temperature rise accompanying sterilization, the important heat labile substances would probably not be damaged. For these reasons, it may be desirable in some instances to substitute radiation sterilization for industrial procedures now in vogue.

The study presented in this thesis was initiated to compare the effects of heat and radiation sterilization of fermentation nutrilites on the yield and rate of lactic acid production. As a basic premise, it was assumed that fermentation nutrilites could be sterilized with ionizing radiations without significant damage. It was further considered probable that, if this could be substantiated, the technique of radiation sterilization would be of value in the preparation of laboratory and industrial media. This would be particularly true in cases where heat sterilization cannot be used and other techniques are too laborious or uncertain.

Some adverse effects of radiation may include the destruction of nutrilites, the formation of toxic substances and the alteration of other important chemical entities. The possible occurrence of such effects on fermentation media was tested indirectly by observing the yield and rate of lactic acid production by fermentation. Since lactic acid organisms have been shown to require a large number of nutrilites (4), their use offers a sensitive test system. Corn steep liquor and malt sprouts were employed, and the results indicated that these sources of fermentation nutrilites can be sterilized with ionizing radiation.

HISTORICAL

1. pH Control and Recording

pH control and adjustment present one of the major problems in microbial fermentations. It is well known that hydronium ion concentration affects bacterial metabolism, and thereby alters the kinds and amounts of fermentation products, as well as their rates of formation (5). The optimum pH range may be quite narrow; for example, a change of 0.5 of a pH unit has been shown to bring about a fourfold change in the rate of product formation (6). Consequently, automatic pH regulation was used in this study because it provided precise control of this variable.

The glass electrode and the automatic pH recorder-controller have greatly facilitated the study of biological systems and are very well adapted for pH control in microbial fermentations. Before the introduction of the glass electrode, no suitable electrode was available for use in bacteriological media. For example, some electrodes required conditions and reagents that affected growth of the organisms. Other suitable electrodes were rendered inaccurate by constituents of the media, and interference from oxidation-reduction potentials often developed. The glass electrode has no effect on the solution or on the medium into which it is placed. When properly installed, it is not affected by stray potentials as it responds only to changes in pH (7) during fermentation.

In 1932 Vickers, Sugden and Bell (8) first used the glass electrode for continuous pH recording. Their apparatus consisted of a glass electrode, a calomel half-cell, a potentiometer and a milliammeter whose reading could be calibrated in pH units.

The first apparatus designed for automatic pH control which was based on the glass electrode was reported by Whitnah (9) in 1933. It consisted essentially of a burette whose operation was indirectly dependent upon pH changes in the medium. The apparatus employed a vacuum tube amplifier, a recording potentiometer and a glass electrode-calomel half-cell system which operated the burette. Whitnah used this equipment to maintain a constant pH during the lactic acid fermentation of milk.

Before automatic pH control apparatus had been developed, Whittier and Rogers (10) had somewhat arbitrarily controlled the pH in the production of lactic acid by fermentation by the continuous addition of hydrated lime at a rate which maintained the pH between 5 and 5.6. They were investigating a continuous fermentation process for the production of lactic acid and reported fermentations with 90 per cent efficiency after two weeks' duration.

In 1935 Longsworth and MacInnes (7,11,12) devised an apparatus to automatically measure and control the pH during the fermentation of a synthetic medium by Lactobacillus acidophilus. Their apparatus depended upon the measurement of the pH with a glass electrode, and control was effected by automatic addition of a sodium carbonate solution. A ninefold increase in acid production and a fourfold rise in the bacterial population was obtained over similar studies without pH control.

For good yields of glycerol during the fermentation of a synthetic medium by Saccharomyces cerevisiae, close control of the pH was found necessary by a group of workers at Iowa State College (13,14,15,16,17).

They employed a Cameron pH recorder that they adapted for automatic pH regulation. Such control increased the glycerol yield by regulating the bisulfite ion concentration.

Leonard, Peterson and Johnson (18) investigated the fermentation of waste sulfite liquors to lactic acid by Lactobacillus pentosus. They maintained the pH at the desired level by the automatic addition of slaked lime or calcium carbonate. The percentage of lactic acid resulting from fermentations buffered with various carbonates was 16 to 80 per cent lower than those obtained with automatic pH control apparatus.

A unique apparatus that would automatically record and control the pH of six different media with only one amplifier was described by Keyes (19) in 1948. The apparatus consisted of a direct current amplifier, a recording potentiometer and a glass electrode-calomel half-cell system. Acid or base was added automatically as needed. Unfortunately, no experiments were reported in which this system had been used.

Breeze (20) has described a precision electronic pH regulating system for the control of biological reactions within ± 0.01 pH units. This complex instrument was a three-channel millivoltmeter with output circuits for the control of valves that permitted the addition of solutions for simultaneously maintaining the desired pH level in three separate fermentations.

The automatic pH control system described by Breeze has been used by Neish and Ledingham (21) and by Watson and co-workers (22) in the 2, 3-butanediol fermentation of a synthetic medium by Bacillus polymyxa or Aerobacter aerogenes. A three- to sixfold increase in the rate of product formation occurred over that obtained in fermentations buffered with calcium carbonate.

In the penicillin fermentation, when the metabolic activities of the mold are in balance, a satisfactory pH is maintained through the steady utilization of lactose. However, the proper balance between nutrilites and products is difficult to maintain. For this reason, Brown and Peterson (23) used an electronic pH controller to operate a valve that allowed the introduction of a neutralizing solution. The yield of penicillin increased markedly and there was a slight rise in the rate of penicillin production. Hosler and Johnson (24) also automatically controlled the pH in penicillin fermentations and have reported similar results.

Kempe, Halvorson and Piret (25) used a commercially-made pH recorder-controller to study the effect of continuously controlled pH on the fermentation of wheat grit media by members of the genus Lactobacillus. They demonstrated that fermentations in which the pH was continuously controlled were equally as effective without the addition of accessory growth factors, as were fermentations with added accessory growth factors and the usual calcium carbonate buffer for pH control.

The same apparatus employed by Kempe was used by Finn, Halvorson and Piret (6) to study the rate of lactic acid formation at various pH levels in the fermentation of dextrose solutions by Lactobacillus delbrueckii. Since the maximum rate of acid production was dependent upon the hydrogen ion concentration, raising the pH only 0.5 units increased this rate as much as fourfold.

Recently, Lakata (26) described a system for the automatic control of pH which was very similar to the one employed in this study. He used a commercial pH meter and potentiometer. Continuous control mechanisms, which could be positioned to operate at any selected pH, were used to add alkali or acid. This system has been used successfully in the

production of citric acid by Aspergillus niger and the enzyme dextranucrase by Leuconostoc mesenteroides.

An apparatus similar to that used by Kempe et al. (25) and Finn et al. (6) was used by Steinberg and Ordal (27) to control the pH during fat production by Rhodotorula gracilis. The rate of fat formation varied linearly with the pH between 3 and 8.5.

2. Mechanism of Action of Ionizing Radiations

Ionizing radiations are any electromagnetic or particulate radiations capable of producing ions, directly or indirectly, in their passage through matter. Two theories on the mode of action of these radiations have been advanced; namely, the "direct action" or "target" theory and the "indirect action" or "activated solvent" theory.

Lea (28) has been the main proponent of the direct action theory which assumes the direct dissipation of energy within a molecule subsequent to collision of an ionizing particle with the molecule. The molecule or structure in which the ionization has been produced is the "target" and the production of ionization in the "target" is a "hit". Therefore, the effect is due to a change produced in a single molecule, the solute molecule. The biological effects to which this theory applies are those in which the effect studied is due to the production of ionization by radiation in, or near, some particular molecule or structure.

In 1929 Risse (29) postulated that the irradiation of water produced atomic hydrogen and OH radicals. Fricke (30), working with aqueous solutions of inorganic and simple organic compounds, and Dale (31,32,33,34,35,36), with enzymes and other biologically active substances, demonstrated that the irradiation of water produced an effect on the solute. They called this intermediate product "activated water". Later,

Weiss (37) proposed that "activated water" contained H atoms and OH radicals. More recently, Dainton (38) has postulated the existence of free radicals and atoms in water and solutions subjected to ionizing radiations.

These developments established the indirect action theory of ionizing radiations based on the formation of intermediate agents which, in turn, act upon the solute. The indirect action theory does not displace the target theory, but rather supplements it.

3. "Dilution and Protection Phenomena" in Radiation of Aqueous Solutions

Dale (31) has studied the effect of radiation on dilute solutions of enzymes and other biological materials. He showed that irradiation of dilute solutions of enzymes caused a greater reduction of activity than that which resulted when concentrated solutions were used. The percentage loss of enzyme activity for a given X-ray dose varied inversely with the initial concentration of the enzyme. This is known as the "dilution phenomenon".

A "protection phenomenon" was also demonstrated by Dale (31). The radiation of carboxypeptidase in the presence of its substrate resulted in some protection of the enzyme against inactivation. This protection has been demonstrated for other biological substances and has been shown to be non-specific, since compounds that could not form an adsorption compound with the solute often provided considerable protection. The degree of protection varied with the concentration and nature of the protecting substance (39,40).

The formation of an active intermediate product from water could explain the protection phenomenon. If the intermediate product encountered two or more different solutes, which were capable of reacting with

the intermediate product, these solutes would compete according to their concentration and nature and leave less material to react with the active solute molecules.

The validity of the dilution and protection effects has been confirmed by many investigators on widely differing biological materials. Among these are viruses (41), bacteriophage (42), toxins (43), enzymes (44), vitamins (45), etc. The references are too numerous to be cited completely but the above-mentioned are important in this connection.

4. Radiation of Culture Media

Lawrence and Graikoski (46) reported that the irradiation of Difco Niacin Assay Medium, to which niacin had been added for the propagation of Lactobacillus arabinosus 17-5, did not result in any change in the medium that could be detected by microbiological assay or visual observations of the growth of the organism.

Elliott, Brownell and Gross (47) have studied the effect of gamma radiation on nutrilites essential for the growth of a protozoan, Tetrahymena pyriformis. Complete, chemically defined, synthetic media were used. These media contained five different groups of components; viz., amino acids, carbon source, nucleic acids, vitamins and inorganic salts.

Tetrahymena grew as well in media whose components were irradiated together in the dry state and then hydrated, as in nonirradiated media. However, complete liquid media, exposed to irradiation, failed to support growth of the organism when irradiation dosages of 5×10^5 rep or more were used. Exposure of complete liquid media to levels of irradiation below 5×10^5 rep allowed the growth of a number of organisms that was directly proportional to the dosage used. This result would be expected on the basis of the indirect action theory, as previously shown with the

enzyme ribonuclease by Lea et al. (41). This enzyme was more resistant to irradiation in the dry state than in solution, because the solute molecules were not exposed to the products of the irradiation of water.

Elliott et al. (47) irradiated the dissolved vitamins independently and added them to the other components of the media which had not been irradiated to determine what fraction was affected. No growth of Tetrahymena was observed in these media. Further work showed that separate irradiation of each of the other media components, followed by their addition to the otherwise complete nonirradiated media, resulted in normal growth of the protozoan. Therefore, they concluded that only the vitamin fraction was adversely affected by the radiation. The addition of irradiated vitamins to a complete medium containing nonirradiated vitamins did not affect the subsequent growth of the organism; so it would appear that the absence of growth was not due to production of toxic products, but rather to partial inactivation of the vitamins.

5. Rates of Fermentation by Lactobacilli

Few detailed rate studies have been reported for the lactic acid fermentation. Longsworth and MacInnes (7,11,12) made a study of the physiology of Lactobacillus acidophilus in media whose pH levels were automatically controlled. The periodic notation of the amount of alkali used to maintain a constant pH value enabled these workers to follow the rate of fermentation. They observed an increase in the rate of acid production up to the end of the logarithmic growth phase, followed by a gradual decrease.

Pan, Peterson and Johnson (3) found that malt sprouts contained a heat-labile growth factor for Lactobacillus delbrueckii which greatly accelerated the fermentation. They followed the rate of acid production

by periodic sugar analyses and obtained a curve showing the percentage of glucose fermented against elapsed time which provided a measure of the rate of fermentation. Only the approximate pH control provided by calcium carbonate buffer was employed in their study.

Kempe et al. (25) noted large variations in the time required for complete fermentation of wheat grit mashes by members of the genus Lactobacillus at various pH levels. An average overall rate was obtained by dividing the amount of lactic acid produced by the time necessary for complete fermentation. It was not possible to measure the instantaneous rates of lactic acid formation in their system.

The effect of continuously controlled pH on the lactic acid fermentation rate was studied by Finn et al. (6). They employed equipment that continuously measured and recorded the instantaneous rate of alkali addition. This was assumed to be directly proportional to the rate of acid formation. They concluded that the rate of lactic acid formation was affected by the pH of fermentation and the quantity of nutrilites used, but that it was independent of sugar concentration.

6. Commercial Production and Uses of Lactic Acid

Lactic acid is of relatively minor importance in industry because of its high cost. It finds greatest use at present in the food industry where lactic acid is employed as an acidifying and preserving agent. It is also used as a deliming agent in the tanning industry and has application in the dyeing of textiles. The amount of lactic acid that can be used by these industries is limited. However, the importance of lactic acid stems from the fact that it has great possibilities as a raw material in other industrial applications.

Fisher and Filachione (48) have shown that lactic acid can serve as a raw material in making various resins, solvents, plasticizers and other industrial products. It is also very versatile chemically and could compete with ethylene and benzene as a basic chemical intermediate.

A recent staff-industry collaborative report on the production of lactic acid from corn sugar (49) has presented an excellent review of the manufacturing process along with a discussion of the various raw materials, methods of purification, marketing grades, economics, production and uses of lactic acid and its derivatives.

METHODS AND MATERIALS

1. Physical Equipment

Continuous automatic pH control was used in this investigation because previous investigators (6,25) have shown that higher rates and yields of lactic acid were obtained when automatic pH control was used in place of the usual calcium carbonate buffer. Manual pH control would yield the same results as automatic pH regulation but would require constant attention.

The pH control apparatus used was of commercial manufacture. It consisted of a Beckman Model RX pH meter, a Bristol Pyromaster Potentiometer and an alkali pump. This system was essentially the same as those described by previous groups (6,25,26). Figure 1 is a diagram of the equipment used for fermentation, pH control and recording the rate of acid formation. Figure 2 is a photograph of the same equipment.

a) pH Meter and Potentiometer

The electrode assembly consisted of a glass electrode, a calomel half-cell, a resistance bulb for automatic temperature compensation and a ground connection for the mash. The potential difference between the glass and calomel electrodes was relayed to an electronic amplifier which amplified and transmitted it to the Bristol potentiometer. This unit continuously recorded and controlled the pH level within ± 0.03 pH units by governing an electric motor that operated the alkali pump which, in turn, removed alkali from a reservoir and added it to the fermenting medium. Considine and Eckman (50) have also described electrodes and incidental equipment of this type.

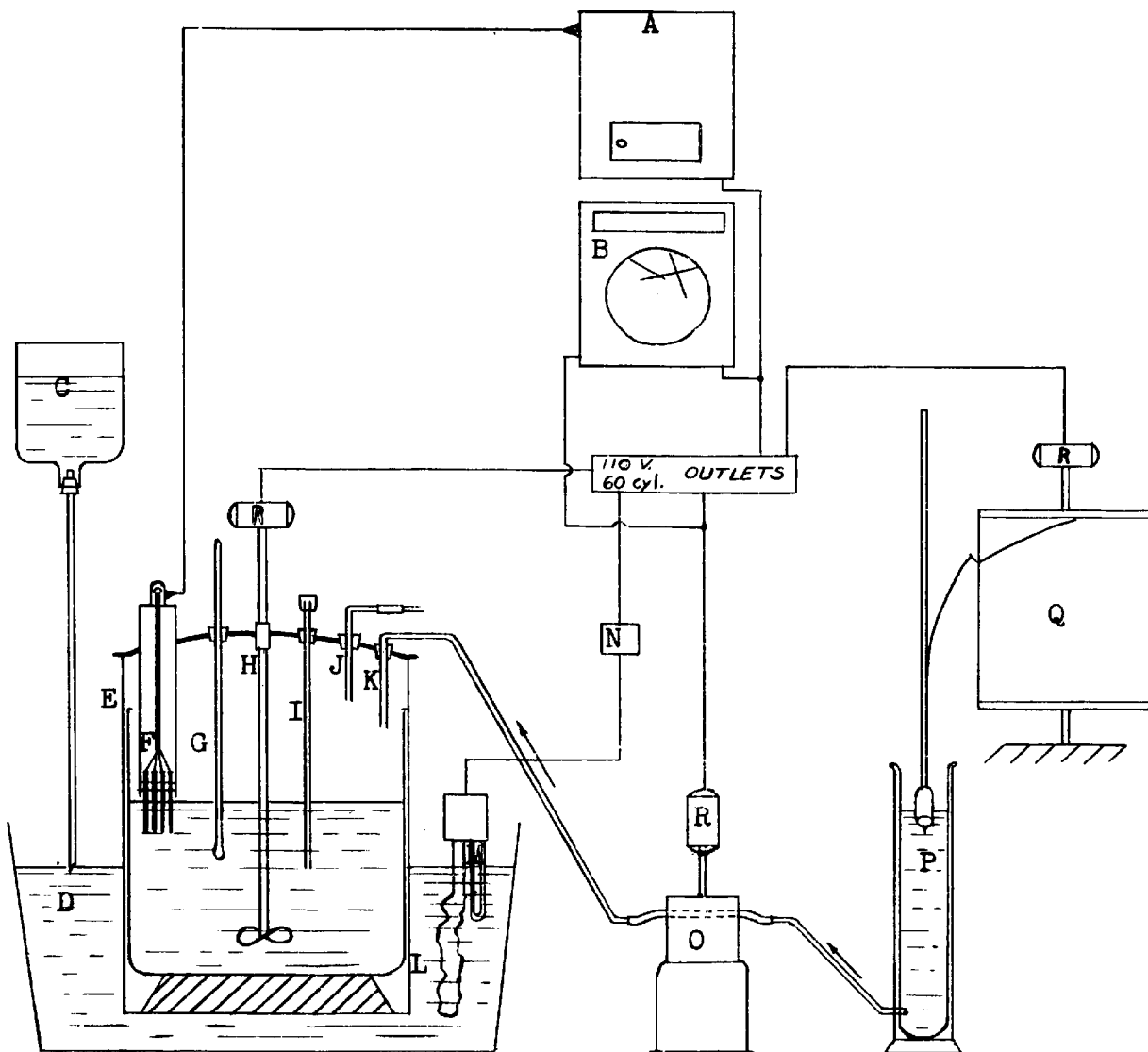


FIGURE 1

DIAGRAM OF EQUIPMENT FOR FERMENTATION, pH CONTROL AND RATE RECORDING

- | | |
|-------------------------------------|---------------------------------|
| A. Beckman pH Meter | J. Optional Addition Line |
| B. Bristol Pyromaster Potentiometer | K. Alkali Addition Line |
| C. Water Level Control System | L. Electric Heater Coil |
| D. Water Bath | M. Thermoregulator |
| E. Fermentor | N. Thermostatic Delay Mechanism |
| F. Electrode Assembly | O. Alkali Pump |
| G. Thermometer | P. Alkali Reservoir |
| H. Agitator | Q. Rate Recording Drum |
| I. Inoculating and Sampling Tube | R. Electric Motors |

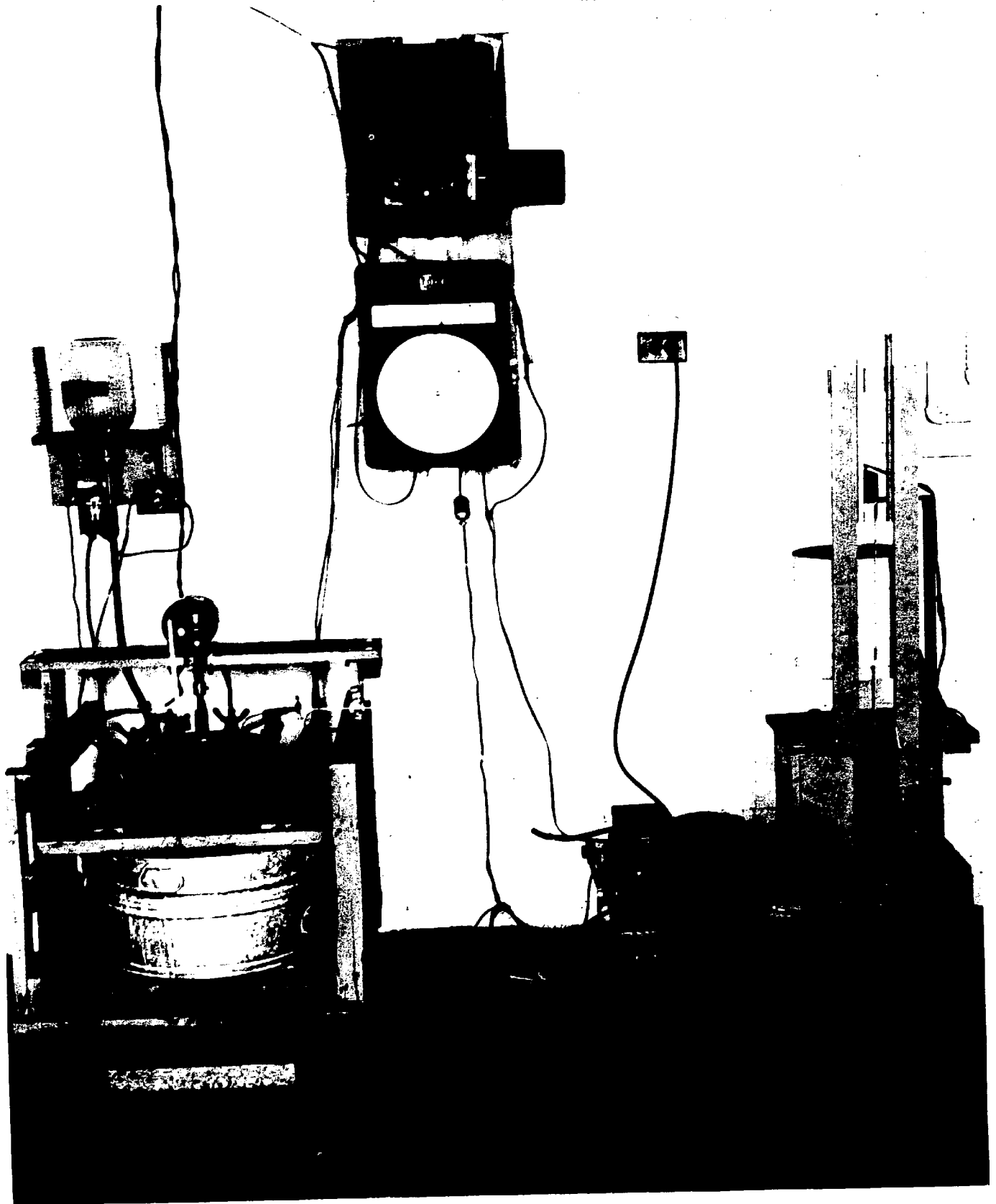


FIGURE 2

PHOTOGRAPH OF EQUIPMENT FOR FERMENTATION, pH CONTROL AND RATE RECORDING

The pH meter was standardized before each run with a 0.5% potassium acid phthalate solution which has a pH of 3.98 at 25°C (51).

The potentiometer recorded the amplified cell potential in pH units on 24-hour charts. In addition, it provided a continuous control mechanism that could be positioned at any selected pH value in the range of 3-10.

b) Alkali Pump

An electrically-driven "Sigmamotor" pump¹ which has no valves and operates on a positive displacement principle was used to introduce the alkali from the reservoir. Metal "fingers" in the pump alternately squeezed the rubber alkali line which had been threaded through the mechanism, and thus forced alkali into the fermenting medium. A rubber check valve placed at the outlet prevented alkali from entering the medium while the pump was not in action. When the pH of the fermenting medium reached the control pH, the motor started and alkali was introduced into the medium from the reservoir. Only a few drops of 3.4N alkali were necessary to raise the pH sufficiently to stop the pump.

The outstanding feature of this pump was the fact that all the parts contacted by alkali consisted of rubber. In this way no foreign ions were introduced into the mash and pump corrosion problems were eliminated. Chlorine water, containing approximately 500 ppm of free chlorine, was used to disinfect the alkali feed line.

c) Rate-Recording Apparatus

Continuous measurements were made of the rate at which neutralizing solutions were added during the course of each fermentation. The principle

¹ Sigmamotor Inc., Middleport, New York.

of the rate-recording device was the same as described by Finn and co-workers (6). It consisted of a kymograph drum, an alkali reservoir, a float mechanism, a balsam shaft and a marking pen. The kymograph drum was set on a ball bearing and rotated once every twenty-four hours by a "Synchron" electric clock motor.² A one-liter graduated cylinder served as the alkali reservoir. The float mechanism was a glass bulb balanced with mercury. The balsam shaft was attached to the float and passed vertically through two metal guides. A marking pen was attached to this shaft in such a way that it would be in contact with the paper covering the surface of the drum. This equipment continuously plotted a fermentation rate curve showing the level of the neutralizing solution in the reservoir as a function of elapsed time.

Withdrawals of as little as 2 ml of neutralizing solution were recorded. The slope of the line on the chart was a measure of the rate of acid production, since at constant pH the amount of acid formed was proportional to the amount of alkali used for neutralization. From analytical data and the fermentation rate curve, the rate of lactic acid production per unit time was calculated.

d) Temperature Control

The temperature of the mash was controlled at 43°C by placing the fermentor in a constant-temperature water bath constructed from a galvanized tub. The temperature of the water in the bath was controlled at 45°C by an electric heating coil and a bimetallic thermoregulator. A thermostatic delay mechanism also was incorporated into this circuit to provide a more precise temperature control by reducing the current

² Hansen Manufacturing Co. Inc., Princeton, Indiana.

that passed through the contact points of the thermoregulator. This prevented the contact points from fusing together.

The water level in the bath was controlled by automatic addition of water from a reservoir above the tub. A glass tube extended from the reservoir to the surface of the water in the tub. When the water level dropped below the end of the tube, water flowed into the tub. It ceased to flow when the end of the tube was covered again.

Since the tub used for the water bath was made of galvanized iron, it soon began to rust. To counteract this, pieces of magnesium rod were suspended in the water to act as sacrificial anodes for rust prevention. Changing the magnesium rods at three-month intervals has prevented noticeable rusting and the water has remained clear. Additional protection against corrosion was provided by the addition of a few milliliters of a saturated solution of sodium dichromate.

e) Fermentor

The fermentor was made from an old vertical autoclave. A pyrex glass battery jar was placed inside the autoclave to serve as the fermentation vessel since lactic acid would corrode the copper which might, in turn, interfere with the fermentations. Water was placed between the pyrex pot and the copper fermentor to aid in the dissipation of heat developed by the fermentation.

The electrodes, thermometer, alkali line and agitator all entered through the top of the fermentor. The agitator was operated at 173 rpm by an electric motor and rapidly dispersed the neutralizing solution in the medium. It was used for this purpose only. The glass agitator was T-shaped with two impellers, each one inch long and 0.25 inches thick. The motor was connected to the agitator shaft by rubber tubing. This

type of connection was provided to prevent overloading the motor in case the agitator shaft should bind. It also permitted easy detachment of the motor. The electrodes, thermometer and electric motor were removed when the fermentor was to be sterilized in an autoclave. Cotton plugs were used to fill holes in the cover provided for these instruments during the sterilization process. The thermometer and electrodes were immersed in chlorine water between runs to prevent contamination. Thus it was possible to operate with an initially sterile system.

A length of glass tubing, covered with a serum bottle cap, extended from the top of the fermentor into the medium and served as a means of adding the inoculum and removing samples for analysis. These operations were carried out aseptically with the use of a previously boiled hypodermic needle and syringe inserted through the serum bottle cap.

f) Grounding of Equipment

It was found to be necessary to carefully ground all equipment to prevent erratic operation. For this purpose, a three-foot length of copper pipe was buried outside the building at a place three feet from the wall and two feet underground. Grounding all equipment to this pipe permitted satisfactory operation.

2. Fermentation Procedure

The constituents of the medium, including glucose, salts, nutrilit source and water, were placed in the glass fermentation vessel where they were mixed. After this, the pH of the mash was adjusted to 6.4 ± 0.1 with sodium hydroxide. The vessel was then placed in the fermentor, the top was attached and bolted, all holes in the cover were

stoppered with cotton plugs, and the entire fermentor along with its contents was autoclaved at 121°C for 45 minutes in a vertical autoclave.

The concentration of the neutralizing solution was 3.4N. Under the conditions of the experimental work, this concentration of alkali was apparently sufficient to kill all microorganisms contacting it since contamination occurred in only two fermentations out of the 88 performed. Furthermore, these fermentations did not appear to have been contaminated by alkali withdrawn from the reservoir. However, as an added safety precaution, the alkali line and reservoir were occasionally sterilized with chlorine water. The charts, one for recording the rate of alkali addition and the other for recording the pH of the fermenting mash, were placed in their respective positions. Then the pH meter was standardized with a 0.5 per cent potassium acid phthalate solution which has a pH of 3.98 at 25°C (51).

After the fermentor was removed from the autoclave, it was placed in the constant temperature bath and allowed to cool to 43°C. The electrodes and the thermometer, which had been standing in chlorine water, were now inserted into the medium through the proper openings in the cover. The alkali line from the reservoir was then connected aseptically to the alkali inlet on the fermentor. Next, the electric motor was attached to the agitator shaft. The agitator bearing was greased with a high-temperature phenolated grease which was added to prevent introduction of contamination by this route. Finally, the agitator and pH meter were placed in operation.

During autoclaving most of the gases, including carbon dioxide, were driven off. Finn et al. (6) found it important to protect dextrose fermentation broths from oxygen during fermentation, although strict

anaerobic conditions were not necessary. For this reason, they introduced carbon dioxide to blanket the surface of the mash during fermentation. In the study reported here the neutralizing solution contained potassium carbonate. When this combined with the sulfuric acid, used for initial pH adjustment, carbon dioxide was evolved and, since a closed system was used, most of this gas remained in the fermentor. No other measures were taken to produce anaerobic conditions.

After the pH of the medium had been readjusted to the level used for the particular fermentation in question, 60 ml of an actively growing culture of L. delbrueckii were added. Next, the pH recording apparatus was placed in operation; the temperature and pH of the mash, and the level of alkali in the reservoir were recorded. Finally, the apparatus for continuously recording the rate of alkali addition to the fermenting mash was connected and placed in operation.

Once placed in operation, the system operated automatically. The pH meter was corrected daily for change in asymmetry potentials to insure precise pH control. Other adjustments were seldom necessary.

Fermentations were considered to be complete when the addition of alkali had ceased. At this time a one hundred milliliter sample was withdrawn from the fermentor. The sample was acidified with sulfuric acid and frozen to prevent further metabolic changes (25). It was then stored in the frozen condition until sugar and lactic acid analyses were carried out. The final volume, pH and temperature of the mash were measured and recorded. The time that the fermentation was completed, the level of neutralizing solution in the reservoir and the amount of the neutralizing solution used were also noted. These data were used to calculate the yield and rate of lactic acid production by the fermentation.

3. Solutions and Media

a) Mineral Salt Solutions

Certain ions have been shown by MacLeod and Snell (52) to be either essential or stimulatory for the normal growth of lactic acid organisms. These ions have been incorporated into the four salt solutions used in this work. These solutions were as follows:

Solution "A"

40 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
 2 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$
 2 g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$
 3 drops HCl
 Distilled water to 200 ml

Solution "B"

50 g Sodium Acetate
 Distilled water to 400 ml

Solution "C"

50 g Na_2HPO_4
 Distilled water to 400 ml

Solution "D"

50 g $(\text{NH}_4)_2\text{HPO}_4$
 Distilled water to 400 ml

All the solutions were stored at 5°C except "C" which was stored at room temperature.

b) Fermentation Mash

The basal fermentation mash consisted of 200 grams dextrose, 10 ml of salt solution "A", 20 ml each of salt solutions "B", "C" and "D" and 3,930 ml of distilled water. In addition, various amounts of corn steep liquor or malt sprouts were added to this basal medium. All the fermentation media were adjusted to $\text{pH } 6.4 \pm 0.1$ before sterilization in order to duplicate, as nearly as possible, the destruction of the

nutrilites by heat. All media were autoclaved at 121°C for forty-five minutes and, after cooling, were adjusted to a pH slightly above that at which the fermentation was to be controlled.

c) Neutralizing Solution

In order that one chart on the kymograph drum would record the rate of acid formation for a complete fermentation, the normality of the alkali solution was established at 3.4N to provide maximum sensitivity. This solution contained equimolar concentrations of sodium hydroxide and potassium carbonate, and approximately 610 ml were required for each fermentation.

d) Fermentation Nutrilites

Corn steep liquor is prepared by concentrating the soluble substances extracted from corn during the preparation of corn starch (53,54). It also contains soluble materials collected at other milling and separating stages. As such, it is an inexpensive, as well as a balanced source of heat-stable fermentation nutrilites. Corn steep liquor contains approximately eight per cent nitrogen. It is high in essential amino acids, minerals and most of the B vitamins. Its main disadvantage lies in the variability between batches. Corn steep liquor can be used as a main source of carbon for the growth of most micro-organisms. It has been used mainly as a source of nutrilites in the penicillin fermentation but has also been used in mashes for the production of lactic acid by fermentation (55).

Malt sprouts result as a by-product in the manufacture of barley malt, which is prepared by germinating the grain under conditions that are conducive to the promotion of the desired physical and chemical

changes. After germination the grain is dried at 50 to 60°C. The rootlets, that are removed at this stage, are called malt sprouts (56, 57). They are used as valuable adjuncts in fermentation mashers and as dairy feed supplements. Malt sprouts are used in mashers for the commercial production of lactic acid by fermentation (3,49).

The corn steep liquor and malt sprouts used were generously supplied by Clinton Foods Inc. of Clinton, Iowa, and the Goebel Brewing Co. of Detroit, Michigan, respectively. For use, the proper amounts were weighed out and added to the basal medium. The mash was then autoclaved at 121°C for 45 minutes. Other samples of the nutrilites were similarly measured and then sterilized by exposure to gamma radiation. These irradiated samples were added aseptically to the heat sterilized basal medium.

4. Organisms and Cultures

A homofermentative organism, Lactobacillus delbrueckii NRRL B-445 obtained from the Northern Utilization Research Branch, United States Department of Agriculture, Peoria, Illinois, was used throughout this study. It fermented glucose almost quantitatively to lactic acid.

Stock cultures were carried as stabs on micro assay culture agar (Difco). They were transferred at three-month intervals. After each transfer, they were incubated at 43°C for 24 to 48 hours and then stored at 5°C in a refrigerator.

The culture in regular use was carried on a liquid medium which consisted of 30 g glucose, 2.5 ml salt solution "A", 5 ml each of salt solutions "B", "C" and "D", 30 g of yeast extract and 1000 ml of distilled water. The pH of this medium was adjusted to pH 6.4 $\pm$ 0.1. Dry calcium

carbonate buffer was then added to each tube before sterilization. This culture was transferred routinely every second day.

The starter culture was grown on a medium of the same composition as the liquid medium described above. These media were inoculated with approximately 5 ml of a 24-hour culture of Lactobacillus delbrueckii and incubated at 43°C for 7 to 8 hours. Sixty ml of such a culture were used to inoculate the fermentor.

5. Gamma Radiation Source

Gamma radiation was supplied by the large cobalt-60 source at the University of Michigan. Its design, installation and operation have been described by Brownell, et al. (58) and Nehemias et al. (59). This source developed 2.2×10^5 rep (roentgens-equivalent-physical) per hour in the center well, while outside the well the dosage rate was a function of the distance from the source. A rep is that amount of radiation which will result in the absorption of 93 ergs per gram of tissue.

An analysis of the radiation field around this source was previously determined by Lewis et al. (60) using the ferrous sulfate method of Weiss (61). Ferrous sulfate dosimeters were incorporated inside some of the corn steep liquor and malt sprout samples being irradiated, in order to determine the amount of radiation absorbed. This information was necessary for the determination of the actual dosage received by these samples.

Samples of malt sprouts and corn steep liquor for irradiation were weighed into round glass containers and exposed to the gamma rays until the desired dosages were received. A radiation dose of 2.5×10^6 rep was employed in this work unless otherwise stated. Small portions of the irradiated samples were placed in appropriate culture media to test for sterility; the remainder of the samples was added aseptically to the mash.

All the samples of corn steep liquor and malt sprouts were found to be sterile after exposure to the ionizing radiation. This was evidenced by the lack of growth on Sabourauds dextrose agar, brain heart infusion broth and veal infusion broth. Observations were made under conditions that were satisfactory for the detection of yeasts, molds, and aerobic and anaerobic bacteria, including spore formers and non-spore formers in the test samples. On the other hand, samples of nonirradiated corn steep liquor and malt sprouts were similarly tested and, in every case, large numbers of sporulating and nonsporulating gram-positive rods were observed upon microscopic examination.

6. Analytical Determinations and Calculations

Previous to a determination, the frozen samples were quickly melted and properly diluted with distilled water. The analyses were made on these diluted samples.

a) Sugar Determinations

Sugar was determined by the method of Shaffer and Somogyi (62) using a conversion factor obtained from calibration analysis made with glucose of analytical reagent grade. This method involved iodometric titrations after a copper reduction. The copper reagent used was described by Somogyi (63) in 1952.

This method is not entirely specific for reducing sugars, and the reducing sugars are not necessarily fermentable; nevertheless, the values obtained by routine analyses of similar menstrums are useful in evaluating the efficiency of fermentations. Somogyi sugar values really represent the reducing capacity of the sample compared to that of glucose.

Potato starch was used to make the indicator solution (64) which was prepared daily. This involved adding a small amount of potato starch

to water, shaking the mixture well, bringing it to a boil, cooling it immediately and then centrifuging. The clear supernatant solution was used. This starch solution yielded a sharp blue endpoint that was easily detected. All other soluble starch solutions tested developed a purple endpoint which was not the true endpoint (65).

A typical calculation for Run 73 will illustrate the manner in which the amount of sugar present after fermentation and the amount of sugar fermented were determined. The amount of fermentable and nonfermentable sugar initially present in the mash was known accurately from the amount of glucose added and from previous analyses of the corn steep liquor and malt sprouts.

Run 73 July 13, 1954

The final volume of the fermentation broth was 4,365 ml. The sodium thiosulfate solution used was standardized against exactly 0.01N potassium iodate and was found to be 0.005N. Ten milliliters of the fermentation broth were diluted to 100 ml and 5 ml of this diluted sample were analyzed. The blank contained 5 ml of distilled water instead of 5 ml of broth.

<u>Blank</u>		<u>Unknown Sample</u>		<u>Difference</u>
25.45	25.50	22.60	22.52	25.48
- 0.00	- 0.00	- 0.00	- 0.00	- 22.56
<u>25.45</u>	<u>25.50</u>	<u>22.60</u>	<u>22.52</u>	<u>2.92 ml</u>
Average: 25.48 ml		Average: 22.56 ml		

The difference in the sodium thiosulfate titration between the blank and the unknown sample was 2.92 ml. This difference, according to the calibration curve, represented 0.425 mg of reducing substance for

the 5 ml analyzed. This was converted into total grams of reducing substance remaining after fermentation, as follows:

$$\frac{0.425 \times \frac{100}{5} \times \frac{4,365}{10}}{1000} = 3.7 \text{ g reducing substance.}$$

The amount of nonfermentable reducing substances in the medium had been determined previously to be 3.4 grams. This amount was therefore subtracted from the total reducing substance after fermentation. Thus $3.7 - 3.4 = 0.3$ grams of fermentable sugar remained after fermentation. The total fermentable sugar initially available was 201.6 grams, being composed of 200 grams of sugar added plus 1.6 grams present in the malt sprouts. The fermentable sugar available (201.6 g) less that present after fermentation (0.3 g) equals the amount of sugar utilized (201.3 g).

$$\frac{201.3}{201.6} \times 100 = 99.9\% \text{ utilization of the available fermentable sugar.}$$

b) Lactic Acid Determinations

The samples were analyzed for lactic acid by the method of Friedemann and Graeser (66) using colloidal manganese dioxide as the oxidizing agent (67). This method depended upon the controlled oxidation of lactic acid to acetaldehyde using colloidal manganese dioxide to control the oxidation-reduction potential. It was necessary to completely oxidize and decarboxylate the lactic acid to acetaldehyde, without further oxidizing the acetaldehyde to acetic acid. The acetaldehyde was removed from the mixture by distillation, in a modified Kjeldahl apparatus, after which it was collected in a sodium bisulfite solution

where it was fixed as an acetaldehyde-bisulfite complex. The excess bisulfite was removed with strong iodine. Then the bound bisulfite was liberated with sodium bicarbonate and sodium carbonate, and the amount present was finally obtained by titration against a standard iodine solution. This method was specific for lactic acid but the presence of proteins and especially reducing substances, like sugars, interfere. The customary precautions were taken to remove the interfering substances by copper hydroxide precipitation. Removal of sugars by $\text{Cu}(\text{OH})_2$ has been studied rather thoroughly by Freeman and Morrison (68) who concluded that, when the bacterial culture medium contained 5 per cent sugar or less, it was not necessary to use more elaborate means than $\text{Cu}(\text{OH})_2$ for removing the sugar. Less than five per cent sugar was present at the end of the fermentations reported here, so copper hydroxide precipitation was considered sufficient for removal of the interfering substances in the mash samples.

Friedemann and Graeser (66) were able to account for 99 per cent of the lactic acid in samples of known composition. However, such good recoveries were not attained with their method in our hands. From many determinations made on two different samples of lithium lactate and one sample of calcium lactate, the recovery of lactic acid was consistently in the range of 85 to 88 per cent, 86.5 per cent being the average value. Consequently, a suitable correction factor was applied to the analytical result in all the analyses reported here. Similar low recoveries on analysis and the subsequent use of a correction factor were reported by Finn (69).

The first problem encountered in the manipulation of the lactic acid determination was the preparation of a reliable oxidizing agent, as the recovery of lactic acid depended upon the quality of the colloidal

manganese dioxide. The first batch that was prepared was not of sufficient concentration to allow maximum recovery of lactic acid. Increasing the concentration of manganese dioxide increased the amount of lactic acid that was detected but, beyond a certain concentration, no further increase in recovery was found. Therefore, a second batch of oxidizing agent was prepared. It was sufficiently concentrated to oxidize all the lactic acid present and thus allowed maximum recovery of the lactic acid.

Lithium lactate, because of its non-hygroscopic nature, was chosen as the standard for the lactic acid determination as recommended by Friedemann and Graeser (66). A sample of lithium lactate was recrystallized using lithium carbonate and reagent grade lactic acid with precautions for the removal of lactides. This sample was redried, and a standard solution of lithium lactate was made for analysis. Repeated analyses of this sample, routinely made in duplicate, accounted for only 85 to 87% of the lithium lactate as lactic acid. Many factors such as errors in standardizing volumetric apparatus, technique of analysis, $\text{Cu}(\text{OH})_2$ precipitation, standard I_2 or oxidizing agent could have been the cause for this low recovery. Re-evaluating and rechecking all of these possibilities revealed no error.

Another sample of reagent grade lithium lactate was obtained for further investigation. Repeated analyses of a standard solution prepared from this source gave good analytical checks, but the same low recovery was obtained.

A standard lactic acid solution prepared from 85 per cent lactic acid was used by Kempe (70). For comparison with results obtained using lithium lactate, a similar lactic acid standard was prepared by boiling lactic acid (reagent grade, 85 per cent) to remove the lactides (71). This acid was standardized by titration against sodium hydroxide of known

normality. Phenolphthalein was used as the indicator to detect the endpoint. Checks on the normality of the solution also were made by potentiometric titrations. Analyses of this lactic acid standard by the Friedemann and Graeser method gave an average recovery of 96.25 per cent of the lactic acid calculated to be present by titration against sodium hydroxide.

A standard sample of calcium lactate, which had been analyzed for calcium and water, was obtained from the California Foundation for Biochemical Research. Analyses of this sample for lactic acid also resulted in 85 to 87 per cent recovery of the lactate expressed as lactic acid.

Thus two samples of lithium lactate and one well-analyzed sample of calcium lactate gave an average lactic acid recovery of 86.5 per cent upon analysis. This evidence indicated that the analytical technique involved a constant error, and that this error would be the same in all samples analyzed. Therefore, a calibration factor, based on the 86.5 per cent recovery, was used for the unknowns. Finn (69) also used a value of 86.5 per cent recovery. Calcium lactate was used as the standard for all subsequent determinations.

The possibility that lactides were present in the lactic acid solution, prepared for use as a standard, could explain the higher apparent recovery of acid upon analysis of that standard. If two molecules of lactic acid were bound together, as is the case in lactylactides, only one free acid group would be available for titration. Upon heating, this combination would break and two molecules of lactic acid would be available for determination. This may well have occurred when the sample was oxidized. Thus more lactic acid could have been present than the titration of the acid with standard alkali had indicated.

The results of the lactic acid analysis of Run 73 will demonstrate the method of calculation.

Run 73 July 13, 1954

The final volume of the fermentation broth was 4,365 ml. A 10 ml sample of this broth was diluted to 1,000 ml with distilled water and 5 ml were used for analysis. The standard iodine solution was 0.01N. Each milliliter of the 0.01N iodine solution, used in the titration of the bound bisulfite, is equivalent to 0.45 mg of lactic acid.

<u>Blank</u>	<u>Unknown</u>	<u>Unknown-Blank</u>
0.05	3.90 3.77	3.83
0.00	0.00 0.00	.05
<u>0.05 ml</u>	<u>3.90 3.77 ml</u>	<u>3.78 ml</u>

Avg.: 3.83 ml

Therefore, 3.78 ml of 0.01N iodine were used to titrate the bound bisulfite and the amount of lactic acid produced was calculated as follows:

$$\frac{3.78 \times .45 \times \frac{1000}{5} \times \frac{4365}{10}}{1000} = 148.5 \text{ g of lactic acid.}$$

The 148.5 grams of lactic acid actually means 171.6 grams were present after correcting for only 86.5 per cent recovery. There were 0.8 grams of lactic acid present in the malt sprouts used, so 171.6 g less 0.8 g equals 170.8 grams of lactic acid produced in the fermentation. All yields reported here are based upon the initial available fermentable sugar which in this fermentation was 201.6 g.

$$\frac{170.8 \text{ g}}{201.6 \text{ g}} \times 100 = 84.8\% \text{ yield of lactic acid.}$$

c) Analyses of Corn Steep Liquor and Malt Sprouts

In order to allow for the fermentable and nonfermentable reducing substances and the amount of lactic acid in the added nutrilites, it was necessary to analyze representative samples of corn steep liquor and malt sprouts for sugar and lactic acid content, before and after a fermentation. For this purpose, fermentations were carried out in the usual manner with the exception that sugar was omitted from the basal medium. Two hundred grams of corn steep liquor or 80 grams of malt sprouts were used in the mash. Samples of the mash were taken before and after fermentation. These samples were acidified and stored in the deep freeze until analyzed as previously described.

The results of these analyses are tabulated in Table 1. Two hundred grams of corn steep liquor contained 12.68 grams of reducing substance of which 9.16 grams were fermentable. Of the 28.3 grams of lactic acid present after fermentation, 19.67 were initially present in the corn steep liquor, while 8.63 grams were produced in the fermentation. Therefore, an average of 94.2 per cent of the fermentable sugar present was converted to lactic acid during the fermentation. These analytical values for corn steep liquor correspond very well with the average values reported by Ligget and Koffler (53).

Eighty grams of malt sprouts contained 6 grams of reducing substance of which 2.6 grams were fermentable. Only 0.8 grams of lactic acid were initially detected in the malt sprouts.

All the glucose and lactic acid present in the nutrilitite sources were corrected for in all the analytical data presented in this thesis.

d) Calculation of the Rate of Lactic Acid Formation

In order to determine the rate of acid formation, it was convenient to measure the rate at which neutralizing solution was added

TABLE 1
THE SUGAR AND LACTIC ACID CONTENT OF REPRESENTATIVE SAMPLES OF CORN STEEP LIQUOR¹
AND MALT SPROUTS² BEFORE AND AFTER FERMENTATION

RUN	SUGAR BEFORE FERMENTATION GMS	SUGAR AFTER FERMENTATION GMS	SUGAR FERMENTED GMS	LACTIC ACID BEFORE FERM. GMS	LACTIC ACID AFTER FERM. GMS	LACTIC ACID PRODUCED GMS
CORN STEEP LIQUOR						
37	12.64	3.52	9.12	20.23	29.44	9.21
38	12.99	3.41	9.58	19.58	27.69	8.11
39	12.41	3.65	8.79	19.20	27.78	8.58
AVG.	12.68	3.52	9.16	19.67	28.30	8.63
MALT SPROUTS						
79	5.8	3.1	2.7	0.7	3.8	3.1
84	6.2	3.7	2.5	0.9	3.8	2.9
AVG.	6.0	3.4	2.6	0.8	3.8	3.0

¹ The values for corn steep liquor represent the amounts contained in 200 grams of corn steep liquor.

² The values for malt sprouts represent the amounts contained in 80 grams of malt sprouts.

since, at constant pH, the amount of acid formed by homofermentative lactic acid bacteria was equivalent to the amount of alkali used for neutralization. This permitted calculation of the rate of acid formation over any interval of the fermentation. Figure 3 is a reduced size drawing of the record chart plotted by the rate-recording apparatus during Run 71. From the slope of this line it can be seen that the rate of fermentation increased rapidly to a maximum, and then gradually declined. For analysis, the fermentation rate curve was divided into one-hour segments. Following this, the rate of alkali addition at each hour was measured with a transparent platen which was calibrated to express the rate of fermentation in terms of milliliters of neutralizing solution added per hour. These values were finally converted into grams of lactic acid formed per 100 ml of medium per hour as described in the following example.

Run 82 July 4, 1954

At the time of maximum rate of acid formation it was found that 45 ml of a 3.4N alkali solution were added to the mash per hour. Therefore, at constant pH, the amount of alkali added multiplied by the normality of the alkali solution and the milliequivalents of lactic acid, equals the amount of acid formed per hour, viz.:

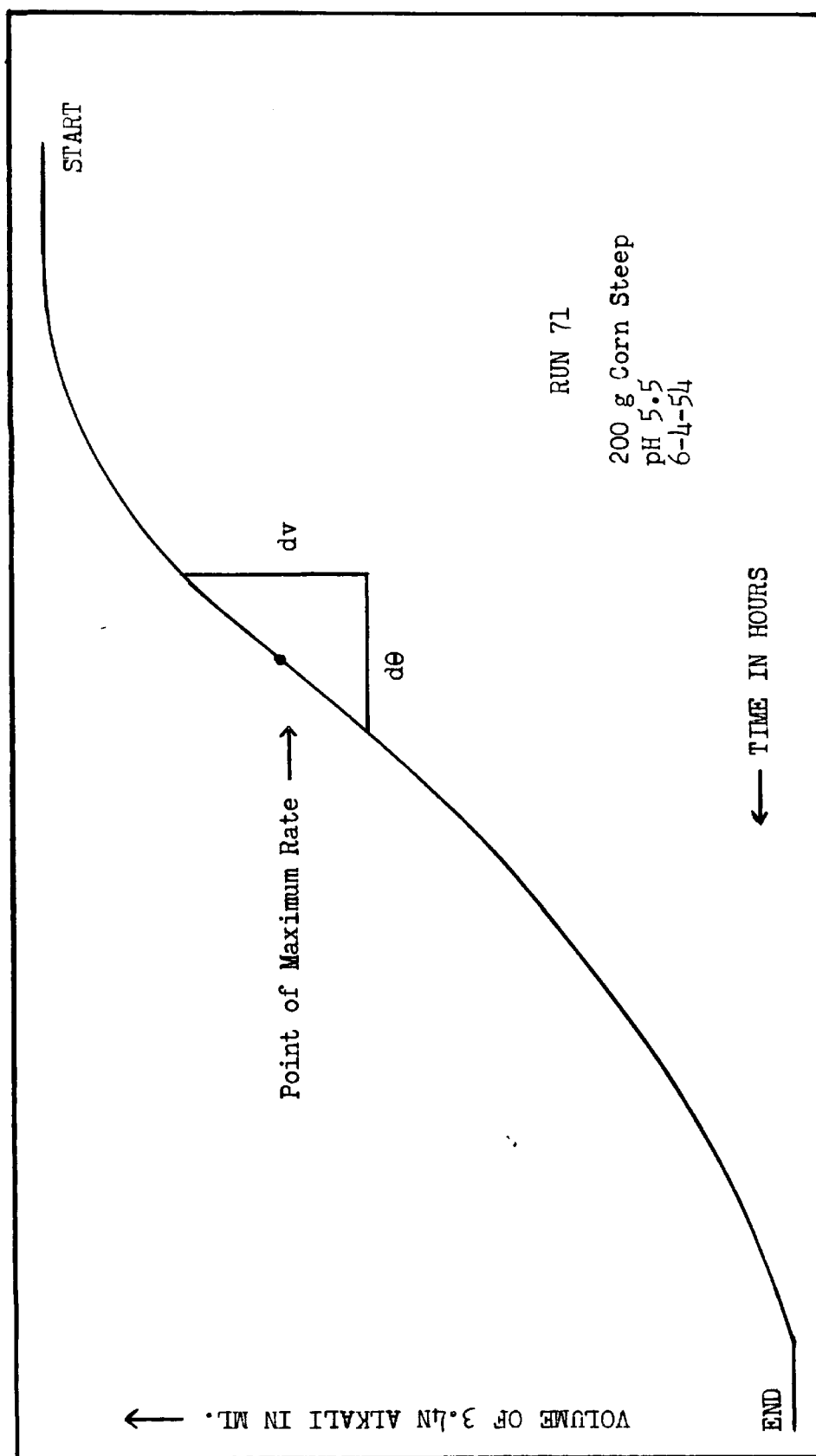
$$45 \times 3.4 \times 0.090 = 13.77 \text{ grams of acid being neutralized per hour at the time of maximum rate.}$$

The volume of the fermenting mash at this time was 4,050 ml.

Thus the amount of acid formed was:

$$\frac{13.77}{4,050} \times 100 = 0.340 \text{ grams per 100 ml of mash per hour at the time of maximum rate.}$$

FIGURE 3
A REDUCED SIZE DRAWING OF THE FERMENTATION RATE CURVE PLOTTED
BY THE RATE-RECORDING DEVICE



This calculation assumed that all the acid neutralized was lactic acid. However, quantitative analyses made at the end of the fermentation showed that only 163.2 grams or 1.813 mols of lactic acid were formed. It is evident that some other acids were formed since the total amount of 3.4N alkali added during the fermentation was 605 ml or 2.057 mols.

Thus:

$$\frac{1.813}{2.057} = 0.881 \text{ mols of lactic acid were formed per mol of total acid neutralized,}$$

hence,

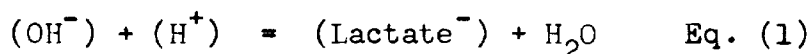
$$0.340 \times 0.881 = 0.300 \text{ grams of lactic acid were formed per hour per 100 ml of mash.}$$

The following theoretical discussion shows that the rates of acid formation, when calculated as shown above, were valid, and that the amount of undissociated acid present in the medium was sufficiently small that it could be neglected. The data used in this discussion was obtained from Run 82 which was carried out at pH 5.5.

Several assumptions were necessary for this discussion. These were as follows: (1) the dissociation constant for lactic acid was 1.38×10^{-4} (77) in the fermented mash; (2) the volume of the fermenting media and neutralizing solution added were accurately known for the time of calculation; (3) the normality of the neutralizing solution was exactly 3.4N; (4) all lactic acid salts were completely ionized after neutralization; (5) lactic acid and other products were formed at parallel rates throughout the entire fermentation; (6) all the acid formed was lactic acid; and (7) hydrogen ion concentration was equivalent to hydrogen ion activity in this solution. A specific calculation, based on these assumptions, is offered as an example.

In Run 82, 0.045 liters of 3.4N alkali were added to the mash per hour at the time of maximum rate of acid formation. Therefore, $0.045 \times 3.4 = 0.153$ mols of alkali were added per hour per total volume of mash. The approximate volume of the medium at the time of maximum rate was 4.05 liters. Thus:

$$\frac{0.153}{4.05} = 0.0378 \text{ mols of alkali were added per hour per liter of fermenting mash at the time of maximum rate.}$$



From the above equation it can be seen that when alkali was added at the rate of 0.0378 mols per hour, 0.0378 mols of acid were being neutralized and 0.0378 mols of lactate ion were being formed per hour per liter.

From the equation for the dissociation constant we obtain:

$$K = \frac{(\text{H}^+) (\text{L}_1^- + \text{L}_2^-)}{N - (\text{H}^+)}$$

Where:

- $N - (\text{H}^+)$ is the undissociated acid in mols per liter.
- (L_1^-) is the lactate ion concentration in mols per liter due to the dissociated acid.
- (L_2^-) is the lactate ion concentration in mols per liter due to the neutralized acid.
- (H^+) is the hydrogen ion concentration in mols per liter at the pH of fermentation. It is also equal to the concentration of the dissociated acid (L_1^-) .

Therefore:

$$N - (\text{H}^+) = \frac{(\text{H}^+) (\text{L}_1^- + \text{L}_2^-)}{K}$$

or:

$$N - (H^+) = \frac{(H^+) (L_1^-)}{K} + \frac{(H^+) (L_2^-)}{K}$$

Now, differentiating with respect to time:

$$\frac{d[N - (H^+)]}{d\theta} = 0 + \frac{(H^+)}{K} \frac{dL_2^-}{d\theta} \quad \text{Eq. (2)}$$

where:

$$\frac{d[N - (H^+)]}{d\theta} = \text{the rate of normality increase of the undissociated lactic acid per hour per liter, or mols of undissociated lactic acid formed per hour per liter.}$$

$$\frac{d(L_2^-)}{d\theta} = \text{the rate of normality increase of the dissociated lactate ion per hour per liter.}$$

$$\frac{d}{d\theta} \left[\frac{(H^+) (L_1^-)}{K} \right] = \text{zero because all three components are constants.}$$

At pH 5.5 the hydrogen ion concentration is 3.16×10^{-6} .

$$\text{pH} = \log \frac{1}{(H^+)}$$

$$(H^+) = \frac{1}{\text{antilog}(-5.5)}$$

$$(H^+) = 3.16 \times 10^{-6}$$

From equation (1) it can be seen that:

$$\frac{d(L_2^-)}{d\theta} = 0.0378 \text{ mols of lactate ion were formed per hour per liter of mash.}$$

Substituting in equation (2):

$$\frac{d[N - (H^+)]}{d\theta} = \frac{(3.16 \times 10^{-6}) (0.0378)}{1.38 \times 10^{-4}} = 0.0865 \times 10^{-2} \text{ mols of undissociated lactic acid formed per hour per liter of mash.}$$

The molecular weight of lactic acid is 90, so:

$$90 \times 0.0865 \times 10^{-2} = 0.0778 \text{ grams of undissociated lactic acid were formed per hour per liter of mash at the time of maximum rate.}$$

At the time of maximum rate of acid formation 0.0378 mols of acid or $90 \times 0.0378 = 3.41$ grams of dissociated acid were formed per hour per liter of mash.

Therefore, the total amount of acid formed per hour per 100 ml of mash was 0.34878 grams. However, it has been previously shown that only 0.881 mols of lactic acid were formed per mol of total acid neutralized. Therefore:

$$0.34878 \times 0.881 = 0.307 \text{ grams of lactic acid were calculated as being formed per hour per 100 ml of fermentation mash according to the previously described assumptions.}$$

The first method of calculation, which neglects that portion of the lactic acid that is undissociated, revealed that 0.300 grams of lactic acid were formed per hour per 100 ml of mash. This means that the undissociated lactic acid accounts for only about 2 per cent of the total at pH 5.5, and thus the undissociated acid can be neglected. Less undissociated lactic acid would be formed at pH 6.2 than at pH 5.5, so it can also be neglected at this pH.

EXPERIMENTAL RESULTS

In order to simplify presentation of the experimental data, the following generalizations are applicable to all the results in this section, except where otherwise indicated. (1) The radiation dosage used was 2.5×10^6 rep. (2) The basal medium contained all the ingredients necessary for fermentation except the nutrilites. (3) Irradiated nutrilites were sterilized independently and then added aseptically to the heat sterilized basal medium at the beginning of each fermentation in which they were used.

1. Effects of Gamma Radiation or Heat Sterilization of Corn Steep Liquor on the Subsequent Production of Lactic Acid by Fermentation

Two series of fermentations were carried out in which the combined basal ingredients and corn steep liquor were sterilized by autoclaving at 121°C for 45 minutes. Corn steep liquor was used initially because a large amount of basic data had been previously collected by the author with this source. In one series the pH was continuously controlled at 6.2; in the other it was similarly controlled at 5.5. Two other series of fermentations were also carried out at the same pH levels. In these, the corn steep liquor was sterilized independently by gamma radiation and then added aseptically to the heat sterilized basal medium immediately before the fermentation was begun. The only variable within each series was the concentration of corn steep liquor employed in the mash. The results of fermentations, in which heat sterilized corn steep liquor was used, served as a base line for the comparison of those results obtained

in fermentations employing corn steep liquor sterilized by irradiation. The experimental results of these fermentations are shown in Tables 2 and 3.

a) Effects on the Rate of Lactic Acid Production

The maximum rates of lactic acid production at the two pH levels are presented graphically as a function of the concentration of corn steep liquor in Figures 4 and 5. When heat sterilized corn steep liquor was used in fermentations at pH 5.5, the maximum rate of lactic acid production increased from 0.20 to 0.594 grams of lactic acid formed per 100 ml of mash per hour as the corn steep liquor concentration in the mash was increased from 1 to 7 1/2 per cent. An increase in the rate from 0.16 to 0.48 grams was also noted when irradiation was substituted for heat. At pH 6.2 the maximum rates of acid production increased from 0.213 to 0.72 grams and from 0.18 to 0.61 grams of lactic acid per 100 ml of mash per hour in fermentations employing heat and radiation treated corn steep liquor, respectively.

In all cases, higher concentrations of corn steep liquor than those necessary to develop the maximum rate of lactic acid production were detrimental. This was evidenced by a decrease in the rate of acid formation. The use of irradiated corn steep liquor instead of heat sterilized for the production of lactic acid by fermentation resulted in a 13 to 20 per cent decrease in the rate of lactic acid formation. A higher rate of lactic acid production was obtained at pH 6.2 than at 5.5, irrespective of the manner in which the corn steep liquor was sterilized. The maximum rates were obtained at the same corn steep liquor concentration in the mash with either sterilizing technique. It was concluded that the maximum rate of lactic acid formation was dependent upon the

TABLE 2
ANALYTICAL RESULTS OF *Lactobacillus delbrueckii* FERMENTATIONS OF CORN STEEP LIQUOR-

GLUCOSE MASHES AT pH 5.5 AND 43°C

RUN	CORN STEEP CONC. USED %	FINAL MASH VOLUME ML	INITIAL SUGAR GMS	FINAL SUGAR GMS	SUGAR USED GMS	LACTIC ACID PRODUCED GMS	SUGAR CONVERSION %	LACTIC ACID YIELD %	MAXIMUM RATE OF ACID FORMATION GM/100 ML/HR (as lactic)	TIME ¹ OF RUN HRS
COMPLETE MEDIA HEAT STERILIZED										
42	2	4,480	203.7	2.95	200.7	200.6	100	98.6	0.273	33
44	3	4,380	205.5	4.20	201.3	195.9	97.4	95.4	0.348	39
31	4	4,660	207.4	8.78	198.6	189.3	95.5	91.5	0.447	18
32	5	4,730	209.2	9.67	199.5	194.2	97.5	93.0	0.488	17
33	6	5,270	211.0	12.33	198.7	192.1	96.8	91.1	0.541	14
34	7	4,850	212.8	13.02	199.8	185.2	92.8	87.0	0.593	13.5
45	8	4,850	214.7	8.92	205.7	193.6	94.2	90.2	0.587	13
43	9	4,820	216.5	8.7	207.8	189.2	91.3	87.3	0.529	13
46	10	4,935	218.3	9.54	208.8	203.8	97.5	93.3	0.553	14.5
CORN STEEP STERILIZED BY IRRADIATION, BASAL MEDIUM WITH HEAT										
60	1	4,070	200.8	4.84	196.0	163.5	83.4	81.4	0.199	53
48	3	4,670	205.5	4.57	200.9	178.2	88.8	86.9	0.253	27.5
71	5	4,625	208.2	5.82	202.4	176.0	86.6	84.6	0.360	19
61	6	4,720	210.0	5.89	204.1	169.8	83.2	80.8	0.455	16.7
72	8	4,800	213.7	6.27	207.4	141.0	67.6	66.1	0.322	17
COMPLETE MEDIUM STERILIZED BY IRRADIATION										
62	3	4,350	205.5	4.59	199.9	168	84.0	82.1	0.138	40
47	6.5	4,130	211.9	3.20	208.7	183.5	88	86.7	0.197	39

¹ The time of run represents the period of fermentation in which 5 ml or more of alkali were added per hour.

TABLE 3

ANALYTICAL RESULTS OF *Lactobacillus delbrueckii* FERMENTATIONS OF CORN STEEP LIQUOR-

GLUCOSE MASHES AT pH 6.2 AND 43°C

RUN	CORN STEEP CONC. USED %	FINAL MASH VOLUME ML	INITIAL SUGAR GMS	FINAL SUGAR GMS	SUGAR USED GMS	LACTIC ACID PRODUCED GMS	SUGAR CONVERSION %	LACTIC ACID YIELD %	MAXIMUM RATE OF ACID FORMATION GM/100 ML/HR (as lactic)	TIME OF RUN ¹ HRS
COMPLETE MEDIA HEAT STERILIZED										
66 ²	8	4,235	214.7	149.0	65.7	43.7	67.6	20.5	—	13.0
52	1	4,390	201.8	2.81	199.0	186	93.5	92.3	0.214	37.5
50	3	4,640	205.5	4.15	201.3	182.4	90.8	88.9	0.415	18
53	4	4,650	207.3	5.00	202.3	179.2	88.8	86.6	0.550	13
22	5	4,630	209.2	8.70	200.5	167.3	83.5	80.1	0.634	11.5
49	6	4,840	211.0	6.58	204.4	180.6	88.4	85.7	0.657	11.25
54	7	4,840	212.8	8.14	204.7	182.2	89.7	85.7	0.712	9.8
CORN STEEP STERILIZED BY IRRADIATION, BASAL MEDIUM WITH HEAT										
56	1	4,530	200.8	3.0	197.8	170.5	86.3	85.0	0.205	44.5
58	3	4,480	204.5	4.3	200.2	173.3	86.5	84.6	0.358	18.25
64	4.37	4,610	207.0	4.2	202.8	171.3	84.2	82.6	0.433	15
59	6	4,730	210.0	4.77	205.2	170.0	83	81.0	0.584	12.5
67	8	4,880	213.7	5.0	208.7	166.2	79.6	77.9	0.590	10.75
COMPLETE MEDIUM STERILIZED BY IRRADIATION										
63	3	4,450	204.5	3.85	200.6	155	77.3	75.8	0.157	30.5

¹ The time of run represents the period of fermentation in which 5 ml or more of alkali were added per hour.² No pH control was used in this run and the final pH was 3.9.

FIGURE 4
MAXIMUM RATES OF LACTIC ACID PRODUCTION IN Lactobacillus
delbrueckii FERMENTATIONS OF CORN STEEP LIQUOR-GLUCOSE MASHES
AT pH 5.5 AND 43°C

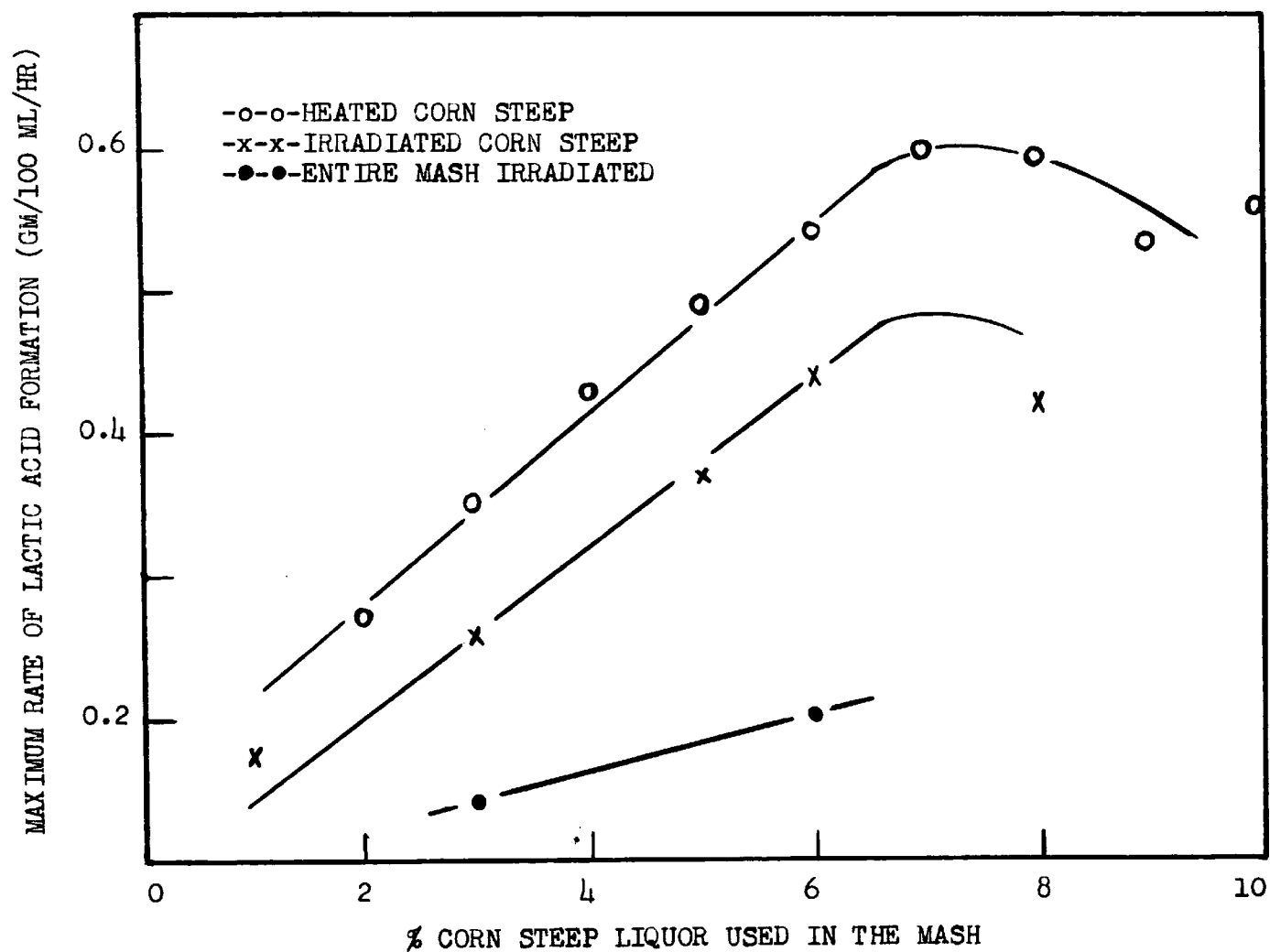
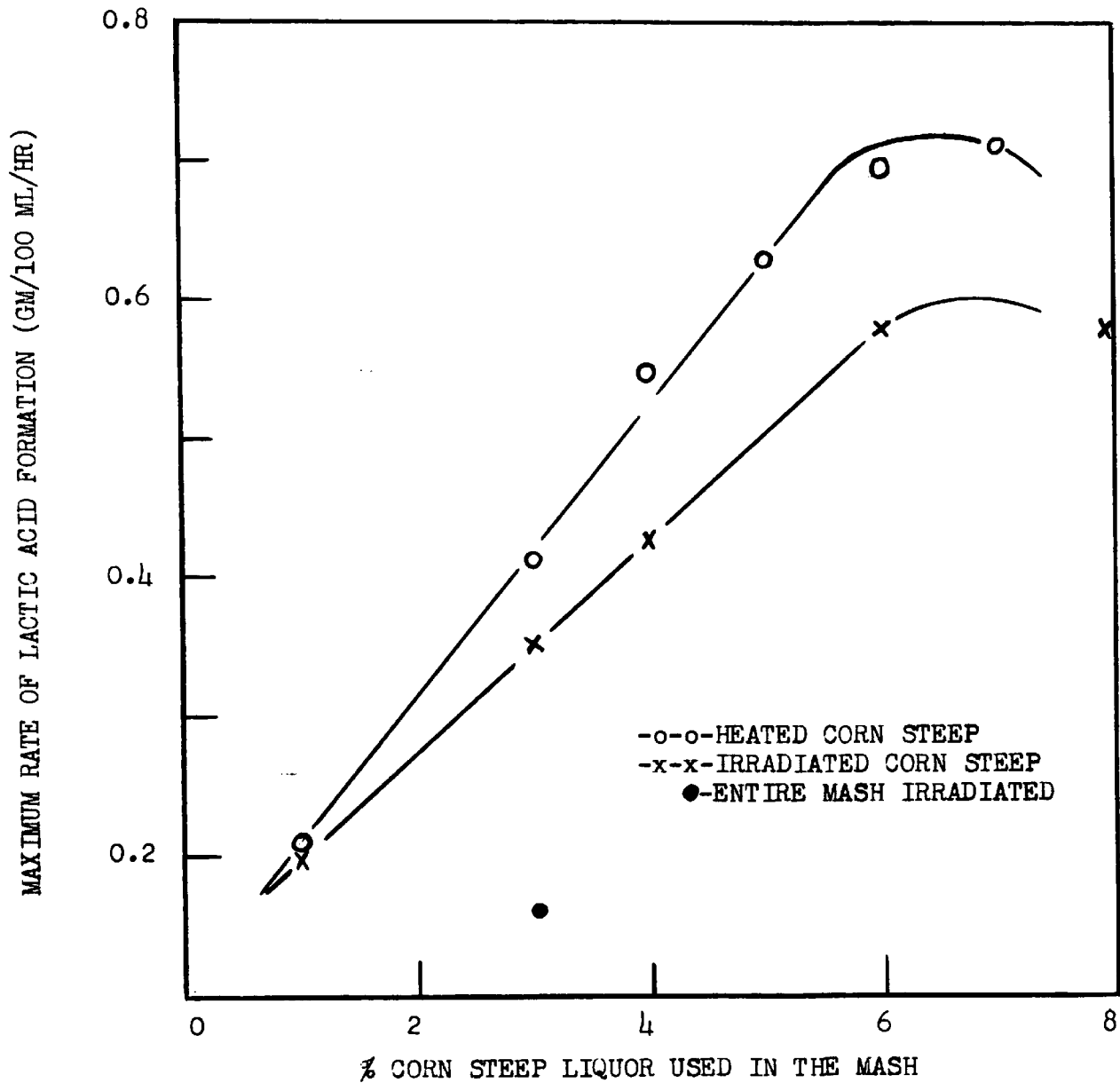


FIGURE 5

MAXIMUM RATES OF LACTIC ACID PRODUCTION IN Lactobacillus
delbrueckii FERMENTATIONS OF CORN STEEP LIQUOR-GLUCOSE MASHES
AT pH 6.2 AND 43°C



concentration of corn steep liquor, the pH at which the fermentations were carried out and the manner in which the corn steep liquor was sterilized.

b) Effects on the Yield of Lactic Acid

The yields of lactic acid obtained are presented graphically in Figures 6 and 7. In fermentations at pH 5.5, the yield of lactic acid decreased from 99 to 85 per cent with increasing concentrations of heat sterilized corn steep liquor. A decrease in yield from 90 to 75 per cent was observed when corn steep liquor sterilized by gamma radiation was substituted for heat treated corn steep liquor. Similarly, at pH 6.2, the yields of lactic acid decreased from 93 to 84 per cent and from 86 to 78 per cent as the concentrations of heat and irradiated corn steep liquor, respectively, increased from 1 to 8 per cent. Fermentations carried out at the higher pH level resulted in a lower yield of lactic acid. This would be expected as higher rates of fermentation are usually accompanied by side reactions that yield other products besides lactic acid (69). A decrease of 6 to 11 per cent in the yield of lactic acid was observed when irradiated, rather than heat sterilized, corn steep liquor was used. Therefore, the yield of lactic acid was dependent upon the pH of fermentation, the concentration of corn steep liquor in the mash and the manner in which the corn steep liquor was sterilized.

c) Effects on the Utilization of Sugar

As shown in Figures 8 and 9, the amount of fermentable sugar utilized decreased, at both pH levels, as the concentration of heat sterilized corn steep liquor in the mash increased. This was true of corn steep liquor concentrations up to, and including, those necessary to obtain the maximum rate of acid production. With higher concentrations

FIGURE 6
YIELDS OF LACTIC ACID IN Lactobacillus
delbrueckii FERMENTATIONS OF CORN STEEP LIQUOR-GLUCOSE MASHES
AT pH 5.5 and 43°C

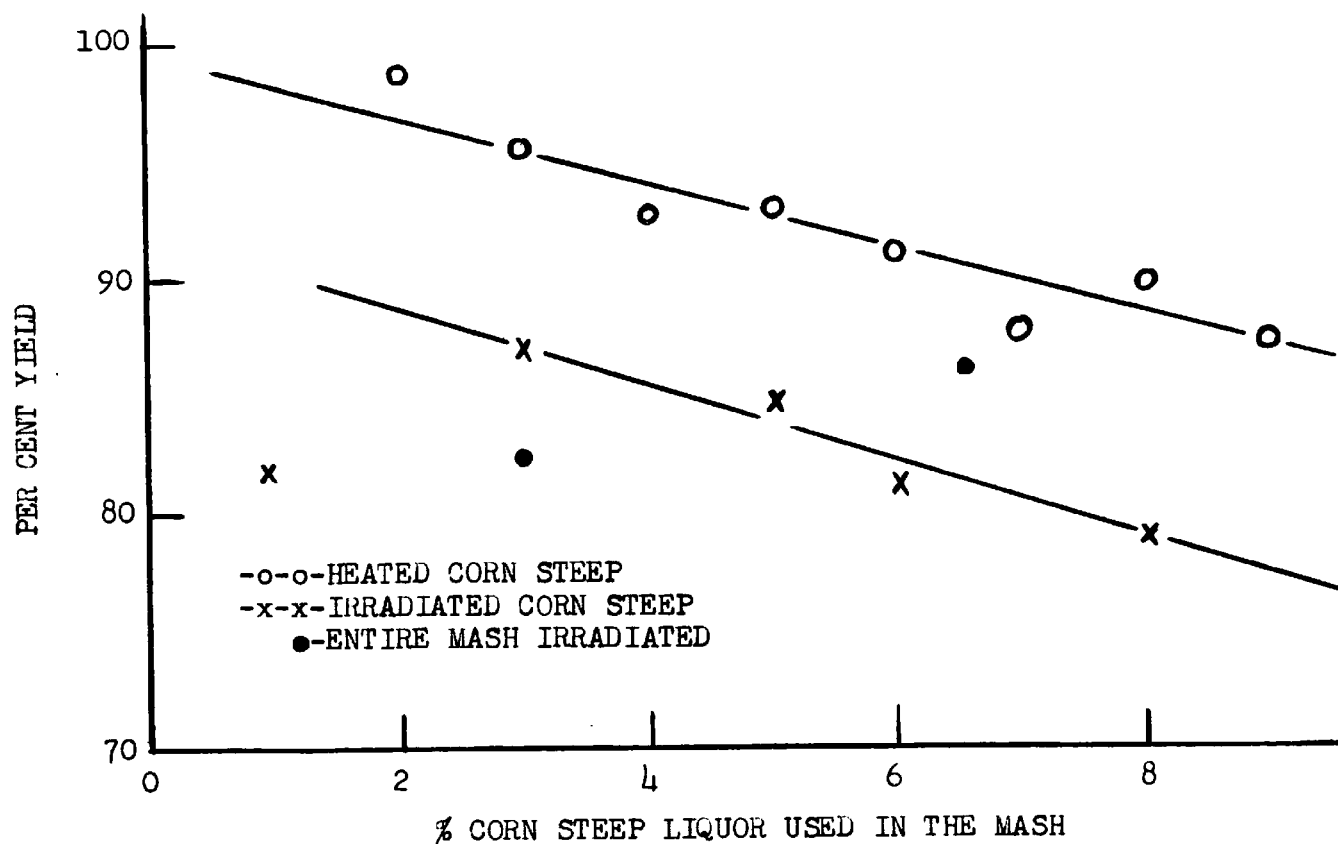


FIGURE 7
YIELDS OF LACTIC ACID IN Lactobacillus
delbrueckii FERMENTATIONS OF CORN STEEP LIQUOR-GLUCOSE MASHES
AT pH 6.2 AND 43°C

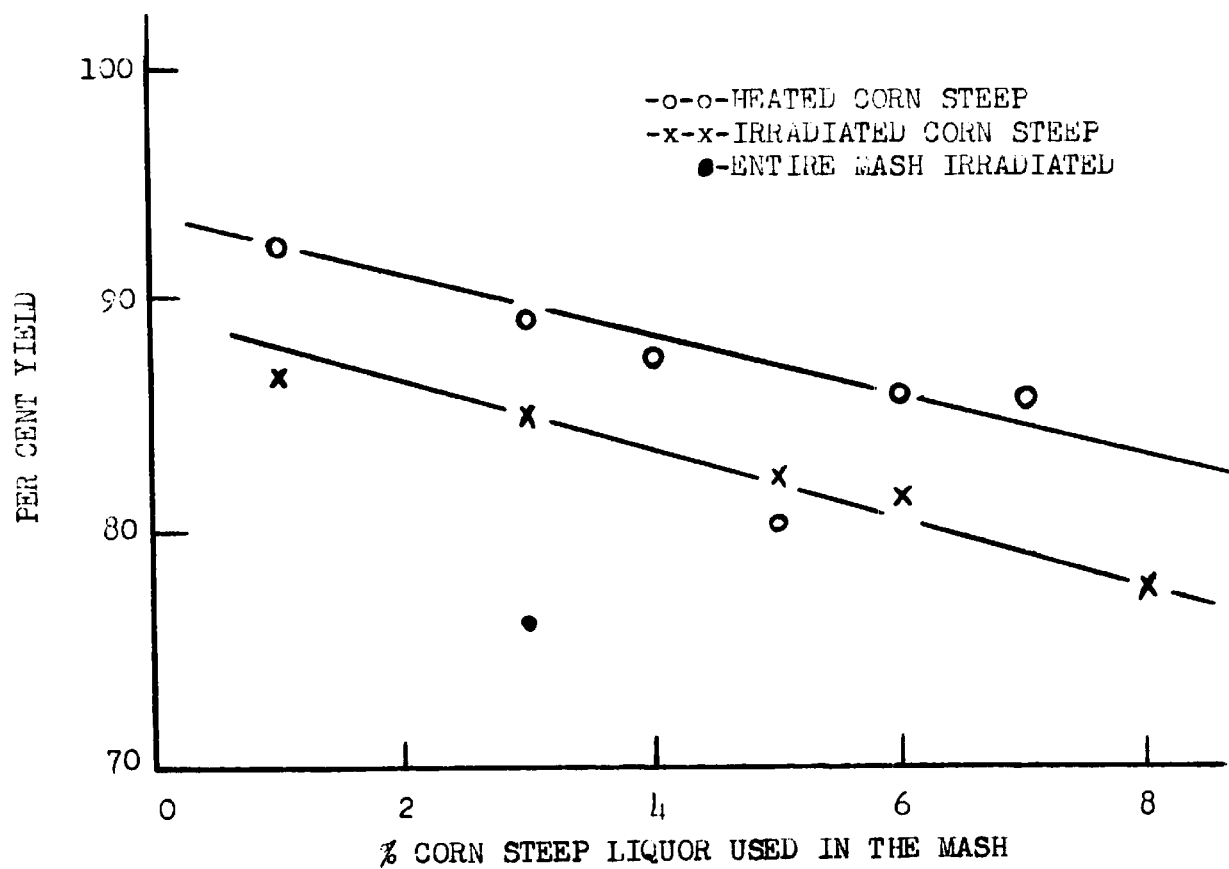


FIGURE 8
 SUGAR UTILIZATION IN Lactobacillus
delbrueckii FERMENTATIONS OF CORN STEEP LIQUOR-GLUCOSE MASHES
 AT pH 5.5 AND 43°C

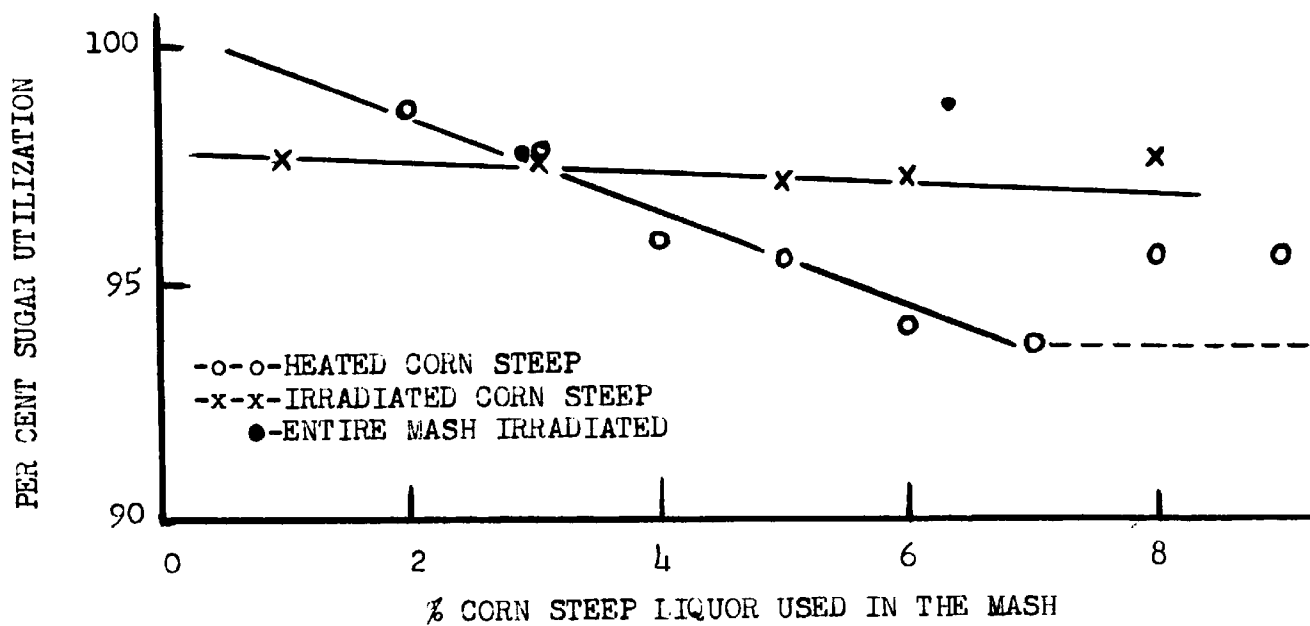
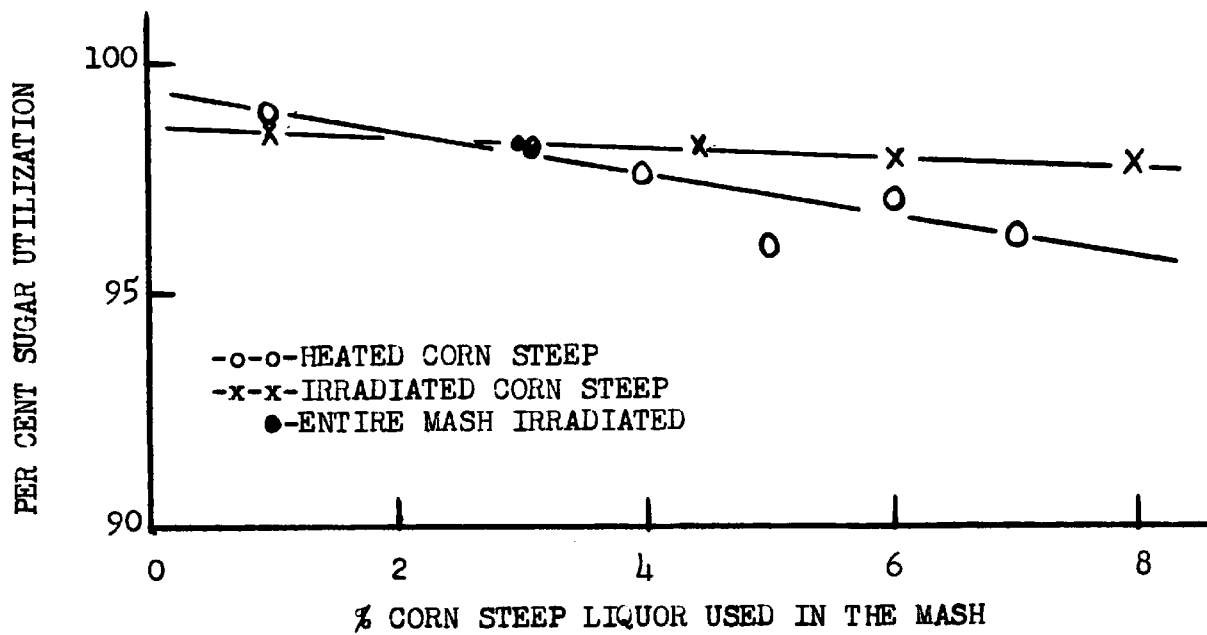


FIGURE 9
SUGAR UTILIZATION IN Lactobacillus
delbrueckii FERMENTATIONS OF CORN STEEP LIQUOR-GLUCOSE MASHES
AT pH 6.2 AND 43°C



of corn steep liquor at pH 5.5, a greater portion of fermentable sugar was utilized. Sufficient concentrations of corn steep liquor were not used in fermentations at pH 6.2 to determine whether this observation was also true at this pH level. However, when irradiated corn steep liquor was employed, about 98 per cent of the available fermentable sugar was used regardless of the concentration of corn steep liquor employed. This constant sugar utilization, coupled with a gradually decreasing yield as the corn steep liquor concentration increased, indicated that the amounts of side products formed were increasing proportionately. In general, the amount of sugar utilized depended upon the pH of fermentation, the manner in which the corn steep liquor was sterilized and the concentration of corn steep liquor in the mash. However, in fermentations employing irradiated corn steep liquor the nutrilit concentration had little or no effect.

d) Effects on Time of Fermentation

The length of time for each fermentation was established as the period during which at least 5 ml of neutralizing solution were added per hour. This avoided errors due to abnormally long lag and declining phases which occasionally occurred when low concentrations of corn steep liquor were used. In the majority of cases the lag period, or period of time after inoculation until 5 ml of neutralizing solution were being added per hour, was of three hours duration at either pH level. The maximum rate of acid production was attained 10 to 12 hours after inoculation in fermentations using heat sterilized corn steep liquor, and 8 to 10 hours after inoculation when irradiated corn steep liquor was used.

The fermentation time for each run decreased rapidly as the corn steep liquor concentration in the mash increased. However, the pattern

of fermentation was the same in all instances. The time necessary to reach the maximum rate of acid production in fermentations in which irradiated corn steep liquor was employed was about 2 hours less than in corresponding fermentations in which heat sterilized corn steep liquor was used. However, the total time of fermentation was about two hours longer in the case of fermentations employing irradiated corn steep liquor, because of the decreased rate of acid formation. The period of fermentation was dependent upon the pH of the fermentation, the corn steep liquor concentration in the mash and, to a lesser extent, on the method used to sterilize the corn steep liquor.

2. Effects of Radiation Sterilization of the Combined Basal Medium and Corn Steep Liquor on the Subsequent Production of Lactic Acid by Fermentation

The results of radiation sterilization of the combined basal medium and corn steep liquor are presented graphically in Figures 4, 5, 6 and 7 and tabulated in Tables 2 and 3. The yields of lactic acid were about the same as those obtained in fermentations in which only irradiated corn steep liquor was used. However, the rate of fermentation was markedly affected. The rate of lactic acid formation at the maximum was 62 to 65 per cent less than the rate obtained when heat sterilized components were used. Thus, irradiation of the entire mash resulted in fermentations with much lower rates of acid production than those developed with heat sterilized mash.

3. Effects of Heat or Radiation Sterilization of Malt Sprouts on the Subsequent Production of Lactic Acid by Fermentation

Malt sprouts are representative of a natural source of fermentation nutrilites. Since they have not been subjected to any heat treatment,

other than that occasioned in drying, they contain most of their initial complement of nutrilites. For these reasons, malt sprouts were employed as the source of nutrilites in the remainder of the experiments.

a) Effects on Rate of Lactic Acid Formation

To parallel the studies using corn steep liquor as the source of nutrilites, several fermentations were carried out at pH 5.5 and 6.2. At each pH level, fermentations employing 2 and 3 per cent malt sprouts which had been either heat sterilized, radiation sterilized or unsterilized were used. Microscopic examinations of slides prepared from samples of the fermented mash were made. No organisms were observed in those mashes in which heat- or radiation-treated nutrilites were used. However, rod-shaped organisms, other than members of the genus Lactobacillus, were observed in the mashes in which unsterilized malt sprouts were used. The results of these fermentations are shown in Tables 4 and 5.

The maximum rates of lactic acid formation, in fermentations employing irradiated malt sprouts, increased from 60 to 75 per cent at pH 6.2 and from 40 to 50 per cent at pH 5.5 when compared to fermentations in which heat sterilized malt sprouts were used. The use of unsterilized malt sprouts in place of heat sterilized malt sprouts increased the rate of acid formation by 48 to 64 per cent at pH 6.2 and by 43 to 45 per cent at pH 5.5.

The rate of lactic acid formation was dependent upon the concentration of malt sprouts in the mash, the pH of fermentation, the manner in which the malt sprouts were sterilized and, to a lesser extent, on the level of radiation used for sterilization.

TABLE 4

ANALYTICAL RESULTS OF *Lactobacillus delbrueckii* FERMENTATIONS OF MALT SPROUT-

GLUCOSE MASHES AT 43°C

RUN	MALT SPROUT CONC. USED %	METHOD OF STERILIZATION	FINAL MASH VOLUME ML	INITIAL SUGAR GMS	FINAL SUGAR GMS	SUGAR USED GMS	LACTIC ACID PRODUCED GMS	SUGAR CONVERSION %	LACTIC ACID YIELD %	TIME ¹ OF RUN ¹ HRS
pH 6.2										
73	2	Heat	4,365	201.6	0.3	201.3	170.8	84.8	84.8	26.5
74	2	Irradiation	4,515	201.6	0.8	200.8	163.8	81.6	81.4	16.5
87	2	Unsterilized	4,505	201.6	0.0	201.6	161.8	80.3	80.3	15.5
75	3	Heat	4,445	202.9	0.0	202.9	159	78.4	78.4	18
76	3	Irradiation	4,575	202.9	0.0	202.9	172.4	84.9	84.9	12
88	3	Unsterilized	4,565	202.9	0.0	202.9	166.8	79.2	79.2	12
80	3	Irradiation ²	4,585	202.9	0.0	202.9	164	80.8	80.8	12.3
pH 5.5										
82	2	Heat	4,415	201.6	3.0	198.6	163.2	82.3	81.2	30.7
78	2	Irradiation	4,245	201.6	2.3	199.3	156.8	78.7	78.0	18
86	2	Unsterilized	4,425	201.6	0.3	201.3	166.8	82.7	82.7	17.5
81	3	Heat	4,485	202.9	0.6	202.3	160.7	79.4	79.2	19.25
77	3	Irradiation	4,535	202.9	0.0	202.9	181.0	89.1	89.1	13.5
85	3	Unsterilized	4,485	202.9	0.0	202.9	164.4	81.1	81.1	12.75
83	3	Irradiation ²	4,505	202.9	0.0	202.9	160.8	79.2	79.2	13.75

¹ The time of run represents the period of fermentation in which 5 ml or more of alkali were added per hour.² Irradiation dosage used was 4.5×10^6 , while all others received 2.5×10^6 rep.

TABLE 5

RATE OF ACID PRODUCTION AND LACTIC ACID YIELD FOR Lactobacillus delbrueckii FERMENTATIONS OF MALT SPROUT-GLUCOSE MASHES AT 43°C

RUN	pH OF RUN	MALT SPROUT CONC. %	METHOD OF STERILIZATION	LACTIC ACID YIELD %	MAXIMUM	INCREASE IN RATE OF ACID PRODUCTION ¹ %
					RATE OF ACID FORMATION GM/100 ML/HR (AS LACTIC)	
73	6.2	2	Heat	84.8	0.313	—
74	6.2	2	Irradiation	81.4	0.542	73
87	6.2	2	Unsterilized	80.3	0.513	64
75	6.2	3	Heat	78.4	0.436	—
76	6.2	3	Irradiation	84.9	0.702	61
88	6.2	3	Unsterilized	79.2	0.645	48
80	6.2	3	Irradiation ²	80.8	0.669	53.5
82	5.5	2	Heat	81.2	0.300	—
78	5.5	2	Irradiation	78.0	0.424	41.5
86	5.5	2	Unsterilized	82.7	0.436	45.5
81	5.5	3	Heat	79.2	0.398	—
77	5.5	3	Irradiation	89.1	0.597	50
85	5.5	3	Unsterilized	81.1	0.570	43
83	5.5	3	Irradiation ²	79.3	0.537	35

¹ Increase in the rate of acid production in fermentations employing irradiated malt sprouts as compared to the rates of acid production in fermentations employing heated malt sprouts.

² Irradiation dosage used was 4.5 million rep, while all other samples received 2.5 million rep.

b) Effects on Yield of Lactic Acid

In all cases 78 to 85 per cent of the available fermentable sugar was converted to lactic acid. Without numerous fermentations, these slight differences in yield cannot be interpreted as significant. Yields obtained in fermentations employing irradiated malt sprouts were comparable to those obtained in fermentations using heat treated or unsterilized malt sprouts.

c) Effects on Other Fermentation Variables

Of the initially available fermentable sugar present, 98 to 100 per cent was utilized in all fermentations in which malt sprouts were used, irrespective of the technique employed for sterilization.

The total time of fermentation was approximately the same for fermentations utilizing unsterilized and radiation sterilized malt sprouts at equivalent pH values and equivalent concentrations of malt sprouts. A much longer period of time was necessary to complete fermentations in which all components had been heat sterilized.

d) Effects of a Higher Radiation Dosage

Two fermentations, numbers 80 and 83, employed malt sprouts sterilized by gamma radiation at 4.5×10^6 rep, while in all the other cases the malt sprouts were sterilized at 2.5×10^6 rep. The use of this higher dosage resulted in a 53 per cent increase in the rate of acid formation at pH 6.2 and a 35 per cent rise at pH 5.5, as compared to rates obtained in fermentations with all heat sterilized components. Hence, approximately doubling the radiation dose decreased the maximum rate of acid formation only slightly. However, the use of this relatively high level of irradiation resulted in much higher rates of fermentation than could be obtained in fermentations utilizing heat sterilized malt sprouts. It would thus appear that a higher level of irradiation does not materially damage the malt sprouts.

DISCUSSION

In order to study the effects of heat and radiation on fermentation mashes, it was considered desirable to utilize a microorganism whose growth response was known to be sensitive to small changes in the nutritive content of the mash. For this reason, Lactobacillus delbrueckii was selected. The requirements for a representative of this species, Lactobacillus delbrueckii strain 730 (ATCC 9649), have been studied extensively by Peters and Snell (4). They showed that this organism required the amino acids alanine, threonine, tyrosine, valine, tryptophan, cystine, serine, aspartic acid, glutamic acid, arginine, lysine, leucine, phenylalanine and histidine for growth. Among the vitamins, calcium pantothenate, nicotinic acid, riboflavin and pyridoxamine phosphate were essential. Pyridoxamine phosphate was not required if both D-alanine and L-alanine were added to an otherwise complete medium. Miscellaneous growth requirements included uracil, hypoxanthine, oleic acid (or other unsaturated fatty acid), thymidine, a fermentable carbohydrate, Mg^{++} , $PO_4^{=}$ and K^+ . Acetate was required for heavy and rapid growth of the organism. The nutritive requirements of the Lactobacillus delbrueckii strain employed in this study were not determined. However, its nutritional requirements were assumed to be sufficiently exacting to detect damage to the nutritives present in the mashes. It was considered probable that such damage would be reflected in changes in yield and rate of lactic acid production.

Some further characteristics of this fermentation and equipment which made it particularly suitable as a test system are outlined as follows: (1) a high percentage of the glucose was converted to lactic

acid, (2) the production of lactic acid was easily followed by observing the amount of alkali used for pH control, (3) aeration and agitation were not required, (4) the fermentations were carried out at a pH and temperature that were not suitable for the growth of most microorganisms, so the possibility of contamination was minimized, (5) reliable pH control equipment was available that would automatically regulate the pH, (6) considerable flexibility of operating conditions was permitted as indicated by the wide pH and temperature ranges available and the rapid pH response to changes in acidity, (7) the rate-recording apparatus plotted a continuous record of the rate of addition of the neutralizing solution as a function of time, (8) sources of fermentation nutrilites were added batch-wise before fermentation or added continuously during the fermentation through separate inlet lines threaded through the pump mechanism, (9) the rate of addition of the fermentation nutrilites was controlled by the same electrical system that governed the addition of alkali, and (10) it was possible to carry out aseptic fermentations.

In the present study the maximum rate of lactic acid formation increased with increasing concentrations of corn steep liquor or malt sprouts in the mash. Previously, Finn (6) had demonstrated that the number of cells, as well as the maximum rate of lactic acid formation, varied directly with the concentration of yeast extract. Consequently, if radiation damaged essential nutrilites in the corn steep liquor or malt sprouts, a decrease in the maximum rate of lactic acid formation should occur. The decrease would depend upon the amount of the nutrilites inactivated which, in turn, would be dependent upon the radiation sensitivity of the essential nutrilites, the radiation dosage used, the concentration of nutrilites and the amount of organic material present in the mash.

The basic premise of this work was that corn steep liquor and malt sprouts could be sterilized with ionizing radiation without significant damage. The experimental results have shown that gamma radiation is more damaging to corn steep liquor than heat. This was evidenced by a 13 to 20 per cent lower maximum rate of lactic acid formation in fermentations in which irradiated corn steep liquor was used. However, it must be noted that corn steep liquor is a by-product of the "wet milling" process and has already been subjected to high temperatures in its production. Such heat treatment would probably destroy most of the heat labile substances. Therefore, based on the assumption that radiation and heat would likely cause different kinds of damage, a decrease in the rate of fermentation would be expected. The evidence supports this view but does not entirely rule out the possibility that the mechanism of inactivation of nutrilites is the same for heat and radiation, with the difference between the two being only quantitative in nature. This suggested the use of a natural source of nutrilites, such as malt sprouts, which have not been damaged by previous heat treatment.

Malt sprouts should contain the heat labile, as well as the heat stable, nutrilites. It is significant that, in this case, heat was much more damaging to malt sprouts than radiation. This was evidenced by an increase in the maximum rate of lactic acid formation from 40 to 73 per cent above that occurring in fermentations in which heat sterilized malt sprouts were used.

Apparently gamma radiation did not materially damage the malt sprouts, since fermentations that were carried out with unsterilized malt sprouts developed even lower rates of lactic acid production than those in which the malt sprouts were sterilized with gamma radiation. Since contaminating microorganisms were found upon routine microscopic examination

of the mashes after fermentations in which unsterile malt sprouts were used, the slightly reduced rate of acid formation in non-sterile fermentations could be due to competition of the contaminating microorganisms for the nutrilites. However, radiation might conceivably enhance the rate of acid production by disrupting some larger molecules, thereby either making some nutrilites more abundant or transforming them into more readily usable forms.

An amount of malt sprouts in excess of that needed to develop the maximum rate of acid production might mask the effect of the ionizing radiation on the essential nutrilites. For example, the inactivated fraction would not be detected if there still remained a sufficient concentration of the nutrilites to develop the maximum rate of lactic acid formation subsequent to irradiation. However, an excess of malt sprouts was apparently never used in this work as was shown by the fact that, when the concentration of irradiated malt sprouts was increased from two to three per cent, there was an increase in the maximum rate of acid formation. This is further substantiated by the observation of a corresponding increase in the maximum rate of acid formation as the concentration of unsterilized malt sprouts was increased from two to three per cent.

Besides the potential use of gamma radiation for sterilizing industrial fermentation mashes, the fact that gamma radiation actually sterilizes media extends its potential bacteriological utility. For example, it seems entirely probable that gamma radiation would be particularly valuable in situations where heat causes undue damage to the nutrilites or other constituents of the media. However, before such uses would be feasible, it was necessary to learn whether sterilization of such media was actually possible. For this purpose, suitable media were inoculated with irradiated samples of corn steep liquor and malt sprouts. This was

followed by incubation under favorable conditions for the growth of all types of microorganisms and the lack of growth under all conditions tested demonstrated that the media had been sterilized. Therefore, it is concluded that gamma radiation can be used to sterilize sensitive nutrilites used in fermentations, and thereby it offers an alternative to the use of heat for this purpose. At the present time, no other practical method is available that will kill all forms of microorganisms. Some methods, such as chemical treatment, ultraviolet irradiation, etc., will remove gross contaminants but are ineffective against bacteriophages, viruses, etc. (1). This really leaves ionizing radiations as the only practical alternative to heat for use in sterilizing culture media for microorganisms.

At present, there are industrial and laboratory fermentations that are not conducted under aseptic conditions. This is not only economical but necessary, since heat would either destroy the nutrilites needed by the organisms employed or would inactivate essential enzymes present in such materials as malt. In general, enzymes are quite resistant to irradiation (31,44). Also, it has been shown in the present study that, in some cases, nutrilites are not significantly affected by ionizing radiations. Consequently, in situations where valuable nutrilites or enzymes may be destroyed by heat, it may prove desirable to substitute irradiation for heat sterilization. Furthermore, the rates of product formation in some biological processes may be increased by the use of irradiation sterilization, as shown here in fermentations employing malt sprouts. Therefore, radiation sterilization could allow increased rates of product formation and the use of aseptic techniques in situations where heat sterilization procedures cannot be employed.

It is fortunate that the damage to organic materials caused by irradiation varies inversely with the concentration of the organic matter,

since in the possible laboratory or industrial applications it would be easier and less expensive to sterilize small volumes of corn steep liquor or malt sprouts with ionizing radiations than to similarly treat large volumes of dilute media. It has been shown here that it is possible to sterilize the combined basal medium and corn steep liquor with gamma radiation. However, this is impractical since there is a marked decrease in the rate of fermentation and some decrease in the yields. Therefore, irradiation of the entire fermentation medium is impractical from the economic, as well as the applied, point of view.

Dale (31) showed that the damage suffered by sensitive biological materials varied indirectly both with their concentration in the medium and the amount of other organic materials present. Corn steep liquor and malt sprouts are complex organic materials. Hence, these should offer a high degree of protection to the nutrilites they contain. This would be especially true if they were not diluted. In such complex materials there are many substances that would protect the nutrilites by inactivating the intermediate products formed during the irradiation of water. The experimental results indicated that Dale's conclusions are applicable to this study. This was based on the observation that a much faster rate of acid formation occurred when the corn steep liquor was irradiated independently than that found when it was irradiated as a component of the diluted mash. This is supported by Elliott et al. (47) who found that, when dilute media were irradiated, they failed to support the growth of Tetrahymena pyriformis. However, when the components of the media were irradiated independently, the adverse effects of irradiation were much less marked.

On the basis of the indirect action theory, the irradiation of fermentation nutrilites in the dry state would cause less destruction of the corn steep liquor and malt sprouts, since they are not exposed to the

products of the irradiation of water. Lea et al. (41) has demonstrated this with the enzyme ribonuclease. Malt sprouts are relatively dry and so the effect of the intermediate products on their nutrilites would be minimal. On the other hand, corn steep liquor is a liquid and so contains an appreciable amount of water. Hence, there should be more destruction of the essential nutrilites in corn steep liquor. This may partially explain the decrease in rate of acid formation when irradiated corn steep liquor was used and the increase in rate when irradiated malt sprouts were employed. It is possible that corn steep liquor could have been dried, then exposed to irradiation, and subsequently used in fermentations without any decrease in rate of fermentation, as compared to those rates obtained in fermentations employing heat sterilized corn steep liquor. While this would have been a useful laboratory experiment, it would have little industrial interest. Hence, no experimental work was conducted to verify this hypothesis.

The plot of the rate of acid formation versus corn steep liquor concentration, as shown in Figures 4 and 5, revealed several facts. For example, progressively increasing rates of acid formation were obtained with increasing concentrations of corn steep liquor. This was observed at both pH levels and with either method of sterilization of the corn steep liquor. Concentrations of corn steep liquor in excess of those necessary to obtain the maximum rate of acid formation were detrimental. This was evidenced by a decrease in the rate of acid formation. The decrease can be explained by assuming that other products were formed at the expense of lactic acid. Also, the threshold concentration of a toxic ion, such as copper, may have been reached, thus adversely affecting the rate. Concentrations of corn steep liquor that are insufficient to allow the development of the maximum rate of fermentation apparently do not

supply enough essential nutrilites. Therefore, at low growth factor concentrations, the rates of fermentation were dependent upon the amount of corn steep liquor supplied.

The maximum rate of acid formation obtained with a given amount of corn steep liquor was dependent upon the pH of the fermentation, as shown in Figures 4 and 5. The higher rate was developed at pH 6.2. This effect is probably the result of the hydrogen ion concentration upon several systems in the aqueous solution, rather than on one system. Doede (72) concluded from a study of the influence of pH upon the growth factor requirements of bacteria that the need for a specific growth factor or the amount required was dependent upon the hydrogen ion concentration. These observations may be interpreted on the basis of the recent work of Simon and Beevers (73) who have made an extensive study of the effect of pH on the biological activities of weak acids and bases. They concluded that, at pH levels below the pK of the weak acids, changes in pH were of little consequence. However, at pH levels above the pK, the adverse activities of the weak acid fall off as much as three times for each pH unit. Aqueous solutions of nutrilites, such as nicotinic acid, may be considered as solutions of weak acids. Consequently, at the higher pH levels less nutrilites would be necessary to develop the maximum rate of lactic acid formation since there would be less adverse effect of the weak acids. Nutrilites are generally constituents of enzymes or associated with them, and thus are intimately concerned in cell metabolism. It is known that most enzymes only operate over a narrow pH range within the cell. Thus, the faster rate of lactic acid production at pH 6.2 may be due to some or all of the following conditions: (1) a more favorable pH for the activity of the enzyme systems involved, (2) a lesser need for external nutrilites than at pH 5.5, (3) a greater ability to use the

preformed source of nitrilites, (4) a faster synthesis and utilization of its own nitrilites, (5) the presence of all the essential nitrilites in the corn steep liquor and malt sprouts and thus no need for synthesis of certain factors that may be rate limiting, (6) a faster and more direct metabolic pathway at this pH than at 5.5, (7) less decay of the nitrilites by the hydrogen ion concentration alone, and (8) a decrease in the adverse effects of the weak acid solutions of nitrilites.

The yields of lactic acid were lower at pH 6.2 than at 5.5. This should be expected because faster rates of fermentation, which were observed at pH 6.2, are usually accompanied by decreased yields. For example, Kempe et al. (25) and Finn et al. (6) obtained the best yields at pH 4.7 and the maximum rate at approximately 5.7 in the lactic acid fermentation. Gunsalus and Niven (5) have shown that, as the pH of lactic acid fermentations increased, the yield of lactic acid decreased. These workers reported that over pH 6.5 the combined yields of other products may account for as much as 25 to 40 per cent of the sugar fermented.

The amount of available fermentable sugar converted to lactic acid in fermentations employing various quantities of irradiated corn steep liquor was constant. This was not analogous to the results obtained when heated corn steep liquor was used. No explanation for this is apparent at present. While this represents one of the differences between the effect of heat and radiation sterilization of corn steep liquor on the subsequent fermentation, it was unimportant for the purposes of this study.

At the present time, the future applications of ionizing radiations for peacetime purposes is uncertain. However, both government and industry are intensely interested in their potentialities. The availability of waste fission products depends upon the continued production of atomic

weapons by the government. Since the disposal of these products is a major problem, their industrial utilization would convert costly waste products into important assets. Furthermore, nuclear reactors are now being erected on some university campuses and industry is seriously considering others for the production of commercial power. These will develop various radioactive by-products that would be useful for experimental and, perhaps, for commercial purposes. The cost of such sources cannot be accurately predicted as yet. Until the economics and several other factors have been further elucidated, it is not possible to predict the future of radiation sterilization even where it may be applicable.

CONCLUSIONS

1. Increases of 40 to 73 per cent in the rate of lactic acid formation occurred when radiation-sterilized malt sprouts were substituted for heat-sterilized malt sprouts in the fermentation mash. This substitution caused no change in the yields obtained.
2. When radiation-sterilized corn steep liquor was used in the fermentation mash in place of heat-sterilized corn steep liquor, the rate of lactic acid formation decreased by 13 to 20 per cent and the yields were reduced by 6 to 11 per cent.
3. It was found more practical to sterilize the nutrient sources independently in the concentrated form rather than as diluted components of the fermentation mash.

APPENDIX

Several problems involving the physiology of members of the genus Lactobacillus were investigated before beginning the study just presented. Since these problems were exploratory and independent in nature, they are described in this appendix. Unfortunately, analytical data for lactic acid per se is not available for many of the fermentations because the samples for analysis were improperly stored. However, in all these cases, the rate and amount of neutralizing solution added were known. Consequently, indirect information concerning the rate of acid formation was obtained.

1. Effects of Various Concentrations of Yeast Extract on the Minimum pH Developed by Lactic Acid Organisms in the Fermentation of Yeast Extract-Glucose Mash

Originally, the effects of various factors on the rate of lactic acid formation under conditions of continuously-controlled pH and temperature were to be investigated. Previous studies by other workers (3,6,18, 25,74,75) had indicated that the rate of acid formation was dependent upon the pH at which the fermentation was carried out and upon the concentration of nutrilites employed in the mash. Therefore, several strains of organisms that convert glucose to lactic acid were investigated to determine the minimum pH at which acid production ceased in the fermentation of yeast extract-glucose mash.

In order to determine the minimum pH developed, several flasks containing 200 ml of sterile media were inoculated aseptically with

cultures of Lactobacillus arabinosus 17-5, Lactobacillus casei, Bacillus coagulans NRRL B-768, Lactobacillus delbrueckii NRRL B-445 and Lactobacillus fermentii. The media consisted of 5 per cent glucose, one milliliter each of salt solutions "A" and "B" and varying concentrations of yeast extract from 0.5 to 25 per cent. After incubation at 37°C for 48 hours, the pH of the contents of each flask was determined and recorded.

As shown in Table 6, a progressively lower minimum pH was observed as the concentration of yeast extract in the media was increased from 0.5 to 3 per cent. However, this minimum pH increased gradually as the concentration of yeast extract was raised from 3 to 25 per cent. In all cases, the amount of decrease or increase was small. The minimum pH attained depended on the organism, but the pattern was the same for all five tested. Consequently, only the results obtained for Lactobacillus delbrueckii are presented graphically in Figure 10.

It was found that, when one to five per cent yeast extract was used in the medium, there was only a slight effect on the minimum pH developed. Also, the minimum pH attained depended upon the organism employed.

2. Fermentations Conducted in an Open Vessel

Since it was easier to carry out fermentations in an open vessel rather than under pure culture conditions, an open-pot type of fermentor was initially employed in conjunction with the pH control and rate-recording equipment described earlier in this thesis. The fermentor consisted of a glass cylinder of about 7-liter capacity. The fermentation mash was prepared and sterilized in flasks, and then added to the vessel at the desired time. The fermentations were carried out in an incubator in order to control the temperature. Only the usual industrial precautions

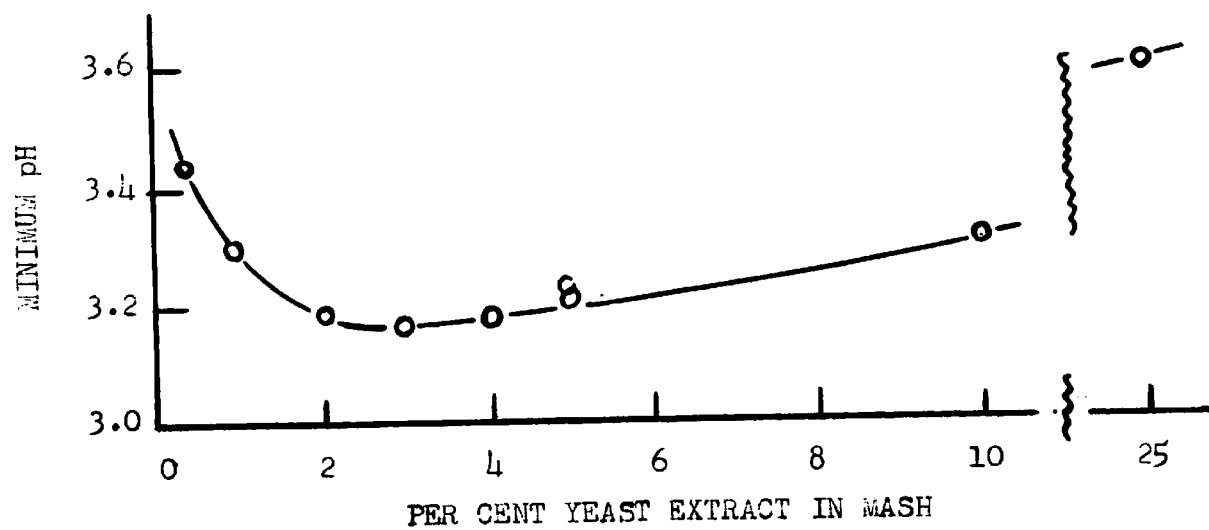
TABLE 6

THE EFFECT OF VARIOUS CONCENTRATIONS OF YEAST EXTRACT ON THE MINIMUM
pH DEVELOPED DURING THE FERMENTATION OF YEAST EXTRACT-GLUCOSE MASHES
BY LACTIC ACID ORGANISMS

ORGANISM	MINIMUM pH							
CONC. YEAST EXTRACT %	0.5	1	2	3	4	5	10	25
<u>L. delbrueckii</u>	3.44	3.31	3.19	3.16	3.17	3.22	3.31	3.59
<u>L. arabinosus</u>	3.50	3.40	3.32	3.30	3.40	3.50	3.60	3.75
<u>B. coagulans</u>	3.52	3.42	3.29	3.25	3.23	3.24	3.32	3.59
<u>L. casei</u>	3.40	3.28	3.15	3.14	3.19	3.21	3.30	3.55
<u>L. fermentii</u>	3.95	3.74	3.47	3.38	3.39	3.40	3.51	3.97

FIGURE 10

THE EFFECT OF VARIOUS CONCENTRATIONS OF YEAST EXTRACT ON THE
MINIMUM pH DEVELOPED DURING THE FERMENTATION OF YEAST EXTRACT-
GLUCOSE MASHES BY Lactobacillus delbrueckii



were employed to control contamination. These were provided by using a high temperature, a large inoculum, a low pH and an initially sterile mash.

After the equipment was in order, several fermentations were carried out to test the complete system. These fermentations were slow and the formation of lactic acid ceased before all the sugar was utilized. During the fourth fermentation a mold growth was detected on the surface of the mash. Various solutions were used to disinfect all equipment in an attempt to prevent the contamination of subsequent fermentations. Unfortunately, this method was ineffective and the amount of mold growth increased with each successive fermentation. The mold was cultured on Sabouraud dextrose agar (Difco) and examined microscopically. It appeared to be similar to Oidium lactis. Consequently, open pot fermentations were abandoned, and the closed system described earlier in this thesis was adopted and used in all subsequent fermentations.

The only information obtained from these fermentations indicated that the rate of acid formation increased to a maximum and then gradually decreased until the sugar supply was depleted.

3. The Effects of Employing Mashs Prepared in Advance on the Subsequent Production of Lactic Acid by Fermentation

Since the effects of various factors on the rate of lactic acid formation were to be investigated, it was considered desirable to ascertain whether the preparation of mashs on one day and their subsequent use on the following day had any effect on the rate of acid formation. This followed from consideration of the possibility that the nutritives in the fermentation mashs had been affected by the hydrogen ion concentration, and that this damage would be reflected in the rate of acid formation.

The considerations were tested by preparing and sterilizing the fermentation mash as usual, and then allowing them to stand at pH 4 or 7 for a period of 24 hours before inoculation. As indicated in Table 7, the maximum rate of acid formation in subsequent fermentations was 12 to 17 per cent less than in similar fermentations that were inoculated immediately after sterilization. This was true when concentrations of one to two per cent corn steep liquor were employed as the source of nutrilites. However, when a concentration of five per cent corn steep liquor was used, little or no difference in the rate of acid formation was observed. Nevertheless, as a result of these experiments, all subsequent fermentation mash was inoculated as soon as they had cooled to 45°C, which required approximately three hours.

It was concluded that some constituents in the corn steep liquor were adversely affected by the hydrogen ion concentration, since the addition of more corn steep liquor increased the rate of acid formation. This observation eliminated the possibility of some toxic substance being responsible for the decrease in rate of acid formation. It would seem that the adverse effects due to the hydrogen ion concentration would have been previously exerted, since the corn steep liquor had a pH of 3.7 in the concentrated form in which it was received. However, the experimental evidence indicates that this was not the case. This could possibly be explained by assuming that the nutrilites which, in many cases, are weak acids (73) were stable in the concentrated form but that, upon dilution, they dissociated and became unstable. This may be analogous to the dissociation of an acid, $HA \rightarrow H^+ + A^-$, where HA is the stable form.

TABLE 7
A COMPARISON OF THE RATES OF ACID FORMATION DURING THE FERMENTATION OF CORN STEEP LIQUOR-GLUCOSE
MASHES BY *Lactobacillus delbrueckii* AS A FUNCTION OF TIME OF INOCULATION AFTER STERILIZATION¹

RUN	CORN STEEP CONC. USED %	FINAL MASH VOLUME ML	INITIAL SUGAR GMS	FINAL SUGAR GMS	SUGAR USED GMS	LACTIC ACID PRODUCED GMS	SUGAR CONVERSION %	LACTIC ACID YIELD %	MAXIMUM RATE OF ACID FORMATION GM/100 ML/HR (as lactic)	TIME ² OF RUN HRS
pH 6.2 22	5	4,630	209.2	8.7	200.5	167.3	83.5	80.1	0.634	11.5
21	5	4,580	209.2	6.8	202.4	170.0	83.9	81.2	0.650	11.7
pH 5.5 42	2	4,480	203.7	2.95	200.7	200.6	100.0	98.6	0.273	33.0
57	2	4,370	203.7	3.2	200.5	195.0	97.3	95.8	0.242	40.0
MAXIMUM RATE OF ALKALI ADDITION ML 3.4N ALK/HR 61 31 24 72										
pH 5.5 15	1	4,280	201.9	—	—	—	—	—	—	61
16	1	4,200	201.9	—	—	—	—	—	—	72

¹ Runs 16 and 21 stood 24 hours at pH 4 and Run 57 stood 24 hours at pH 7 before they were inoculated. The other three runs were inoculated immediately after sterilization.

² The time of run represents the period of fermentation in which 5 ml or more of alkali were added per hour.

4. Effects of the Mode of Addition of the Source of Nutrilites on the Subsequent Production of Lactic Acid by Fermentation

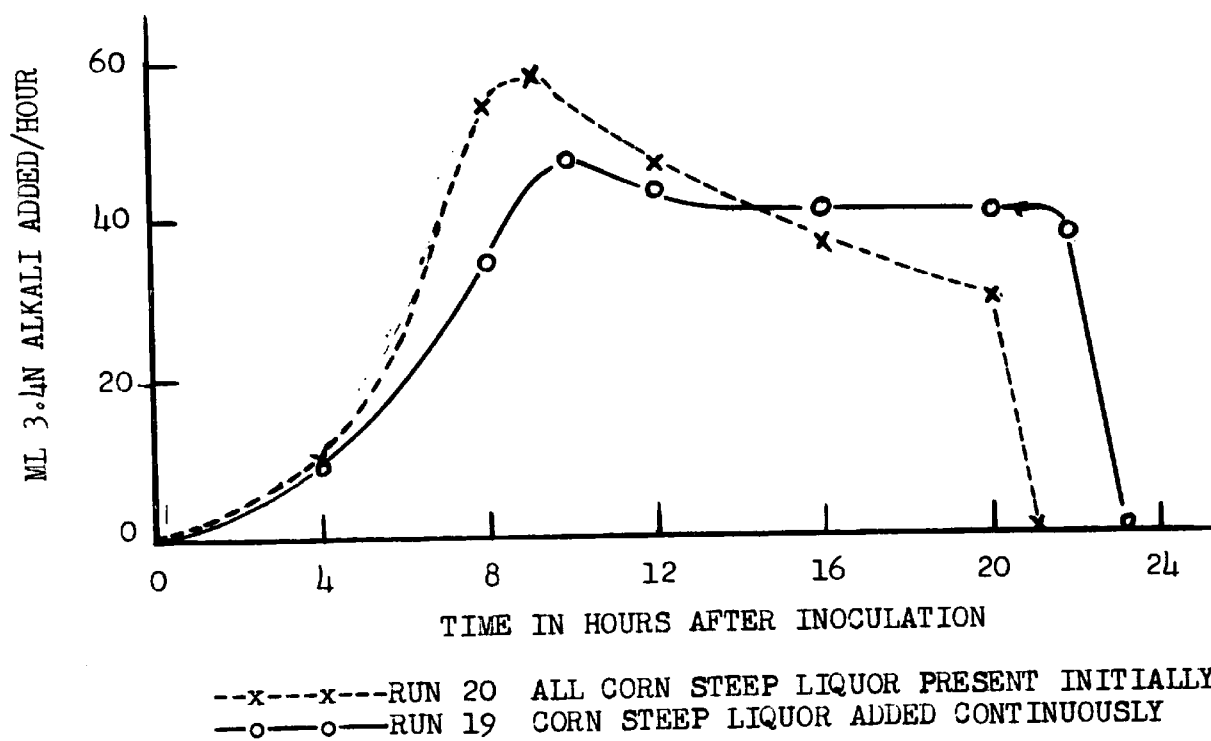
In the early fermentations it was observed that the rate of addition of the neutralizing solution increased rapidly to a maximum and then gradually decreased. The graphic representation of the rate of addition of neutralizing solution against time is presented in Figure 11. The broken line represents a fermentation in which all the corn steep liquor was present initially. The solid line represents a fermentation in which a portion of the corn steep liquor was added continuously. However, the total amount of corn steep liquor used was 3 per cent in both cases. In an industrial fermentation the long declining phase is costly. Consequently, if a constant rate of fermentation could be maintained after the rate had attained its maximum, a saving in time and cost would result. Therefore, it was considered desirable to determine if the declining phase could be altered.

After the rate of acid formation had noticeably decreased in one of these fermentations, corn steep liquor was added to the fermenting mash. An immediate increase in the rate of acid formation was observed. This was followed by another maximum and then it gradually declined as before. This observation was repeated several times by adding corn steep liquor, yeast extract or yeast water to the fermenting mash when the fermentation was in the declining phase. The yeast water was prepared according to the method of Wilson and Knight (76).

This observation, coupled with the previous indication that some constituents of corn steep liquor were adversely affected by the hydrogen ion concentration, suggested that the continuous or periodic addition of a source of nutritives during the fermentation might be advantageous. It was considered probable that the continuous addition of corn steep liquor

FIGURE 11

THE EFFECT OF CONTINUOUS ADDITION OF CORN STEEP LIQUOR ON THE
 RATE OF ALKALI ADDITION IN THE FERMENTATION OF CORN STEEP
 LIQUOR-GLUCOSE MASHES BY Lactobacillus delbrueckii AT pH 6.2



during fermentation might result in a constant rate of acid formation.

If this assumption could be substantiated, there would be several advantages to using the continuous addition of corn steep liquor. (1) In fermentations that are prone to develop side reactions the initial concentration of corn steep liquor in the mash could be decreased and, consequently, a slower but more constant rate of acid formation would result. The overall time of fermentation probably would not be significantly affected. (2) The yield of lactic acid would be increased, since it was known that higher yields were obtained when smaller amounts of nutrilites were used. (3) The costly declining phase would be eliminated. (4) The length of time necessary to complete a fermentation might be reduced. Therefore, an investigation of these possibilities was initiated.

For this purpose, the corn steep liquor which was to be added continuously during a fermentation was prepared by mixing it with a suitable volume of water. The pH was then adjusted to approximately 0.2 units above the control pH employed for that particular fermentation, and the mash was sterilized by autoclaving in a dispensing funnel for 20 minutes at 121°C. In order that it could be pumped into the fermenting mash, one end of a length of sterile rubber tubing was aseptically attached to the dispensing funnel. The other end was threaded through the pump mechanism that added alkali and was then attached to the inlet line on the fermentor. Consequently, every time alkali was added to control the pH, corn steep liquor was simultaneously introduced.

The results of fermentations, in which continuous addition of the source of nutrilites was employed, are tabulated in Table 8. A graphic plot of the rate of addition of neutralizing solution as a function of time is presented in Figure 11. The total concentration of corn steep

TABLE 8
THE EFFECT OF THE CONTINUOUS ADDITION OF NUTRILITES ON THE RATE OF ALKALI ADDITION IN THE
FERMENTATION OF CORN STEEP LIQUOR-GLUCOSE MASHES BY *Lactobacillus delbrueckii*

RUN	CORN STEEP CONC. USED % I ¹ C ²	FINAL MASH VOLUME ML	INITIAL SUGAR GMS	FINAL SUGAR GMS	SUGAR USED GMS	LACTIC ACID PRODUCED GMS	SUGAR CONVERSION %	LACTIC ACID YIELD %	MAXIMUM RATE OF ACID FORMATION GM/100 ML/HR (as lactic)	TIME ³ OF RUN ³ HRS
pH 6.2										
23	5	5,000	609	21.0	588	508	86.5	83.6	0.515	32
25	3	5,350	619	20.0	589	518	88.0	83.8	0.636	28
40	1	5,950	409 ⁴	3.0	406	399	98.3	97.6	0.327	34
pH 5.5										
35	7	5,940	413	11.7	401	387	96.5	93.8	0.487	39
36	7	6,240	422	11.4	410	380	92.5	90.1	0.482	28
pH 5.5										
17	1	4,350	201	—	—	—	—	—	18.0	41
18	2	4,600	201	—	—	—	—	—	23.5	36
pH 6.2										
19	1	4,500	205	—	—	—	—	—	46.0	20
20	3	4,640	205	—	—	—	—	—	60.0	18

MAXIMUM
RATE OF ALKALI
ADDITION
ML 3.4N ALK/HR

¹ Corn steep liquor initially present in the mash.

² Corn steep liquor added continuously.

³ The time of run represents the period of fermentation in which 5 ml or more of alkali were added per hour.

⁴ Only 200 g of sugar were present initially with 100 g being added after 15 hours and another 100 g after 27 hours.

liquor used in these two fermentations was the same. In Run 20, indicated by the broken line, a three per cent concentration of corn steep liquor was initially present in the mash and none was added during the fermentation. One per cent corn steep liquor was present initially in Run 19, indicated by the solid line, and two per cent was added continuously.

In Run 19 the continuous addition of the nutrilites allowed a constant rate of acid formation to be maintained. In general, the concentration of nutrilites present in the initial mash and the pH of fermentation determined the maximum rate of acid formation developed. This was true regardless of whether the nutrilites were added continuously or not. It can also be seen that when corn steep liquor was added continuously, the rate of acid formation increased rapidly to a maximum. Then, instead of decreasing gradually as was the case when all the nutrilites were present initially, the rate decreased slightly to a level where it remained constant until the sugar concentration became the limiting factor. The maximum rate of acid formation developed was significantly greater when all the corn steep liquor was initially present, but the overall time was nearly the same. If a higher concentration of sugar had been employed, it is likely that the declining phase would have been much longer and, consequently, the time required to complete the fermentation would have been shorter in fermentations in which the continuous addition of nutrilites was employed. Comparative data was not obtained to demonstrate this view.

A constant rate of acid formation could not be maintained when the initial concentration of corn steep liquor was five per cent or higher. Thus, the general shape of the curve was the same whether the corn steep liquor was present initially or added continuously.

Consequently, there was no advantage in the continuous addition of nutrilites in these fermentations.

It was concluded that the continuous addition was advantageous in those fermentations in which low concentrations of corn steep liquor were employed, but was unimportant when high concentrations of corn steep liquor were used.

5. A Possible Commercial Application of the Continuous Addition of Nutrilites

The fact that a constant rate of fermentation could be maintained under certain conditions by the continuous addition of corn steep liquor suggested the possible application of this technique to a continuous fermentation process for the production of lactic acid. A single fermentation, Run 40, was carried out to test this consideration. These results are included in Table 8. Initially, the fermentation mash contained 5 per cent sugar (200 g) and one per cent corn steep liquor (40 g). Another four per cent of corn steep liquor (160 g) was added continuously during the fermentation. In this run the rate of acid formation increased rapidly to a maximum about ten hours after inoculation and remained constant at this maximum rate. Fifteen hours after inoculation, five per cent or 100 g of glucose were added; immediately, the rate of acid formation began to increase slowly to a new maximum which was attained after 25 hours of fermentation. Another 100 g of glucose were added at 27 hours, and no effect was observed on the rate of acid formation. However, after 32 hours of fermentation the addition of corn steep liquor was stopped. This was followed by a gradual decrease in the rate of acid formation until the sugar was depleted, which occurred 38 hours after the fermentation was begun. In this fermentation 99.5 per cent of the available fermentable

sugar was utilized, and 97.6 per cent of the amount available was converted to lactic acid. A maximum rate of lactic acid formation of 0.327 grams per 100 ml per hour was developed.

This was not a true continuous fermentation since none of the fermentation mash was withdrawn. However, the results suggested that an efficient system of this nature could probably be developed based upon the information obtained from this experiment.

6. The Effects of Sugar Concentration on the Subsequent Production of Lactic Acid by Fermentation

Finn (69) observed that the sugar concentration in the medium had no effect on the rate of acid formation. This was true in those cases where the sugar concentration was not the limiting factor. It is evident that a higher sugar concentration would increase the time necessary to complete a fermentation. This would result in a longer period of time in which to study the effect of the continuous addition of corn steep liquor on the rate of acid formation. Consequently, it was considered desirable to test the effect of sugar concentration on the rate of acid formation.

The experimental results, shown in Table 9, indicated that the sugar concentration decreased the rate of maximum acid formation by 10 per cent as the sugar concentration in the mash was increased by 5 per cent. Also, the length of the lag period was increased by eight hours when the sugar concentration was increased from 5 to 15 per cent.

Insufficient data was collected to draw accurate conclusions in this regard. Also, it is recognized that the decreased rate of acid formation with the higher sugar concentrations could be due to an osmotic pressure effect.

TABLE 9
THE EFFECT OF SUGAR CONCENTRATION ON THE RATE OF ACID FORMATION IN THE FERMENTATION OF
CORN STEEP LIQUOR-GLUCOSE MASHES BY Lactobacillus delbrueckii

RUN	CORN STEEP CONC. USED %	FINAL MASH VOLUME ML	INITIAL SUGAR GMS	FINAL SUGAR GMS	SUGAR USED GMS	LACTIC ACID PRODUCED GMS	SUGAR CONVERSION %	LACTIC ACID YIELD %	MAXIMUM RATE OF ACID FORMATION GM/100 ML/HR (as lactic)	TIME ¹ OF RUN HRS
pH 6.2										
22	5	4,630	209	8.7	200.5	167.3	83.5	80.1	0.634	11.5
23	5	4,990	609	21.0	588.0	508.5	86.5	83.5	0.515	32
pH 5.5										
33	6	4,850	211	12.3	198.8	192.1	96.8	91.1	0.541	14
35	6	5,240	411	11.7	399.3	387.0	96.8	93.2	0.487	38

¹ The time of run represents the period of fermentation in which 5 ml or more of alkali were added per hour.

BIBLIOGRAPHY

1. Pfeifer, V. F., and Vojnovich, C., 1952, Continuous Sterilization of Media in Biochemical Processes, Ind. Eng. Chem., 44:1940.
2. Williams, R. J., 1941, Growth-Promoting Nutrilites for Yeasts, Biol. Rev., Cambridge Philosophical Society, 16:49.
3. Pan, S. C., Peterson, W. H., and Johnson, M. J., 1940, Acceleration of Lactic Acid Fermentation by Heat-Labile Substances, Ind. Eng. Chem., 32:709.
4. Peters, V. J., and Snell, E. E., 1954, Peptides and Bacterial Growth. VI, The Nutritional Requirements of Lactobacillus delbrueckii, J. Bact., 67:69.
5. Gunsalus, I. C., and Niven, C. F., Jr., 1942, The Effect of pH on the Lactic Acid Fermentation, J. Biol. Chem., 145:131.
6. Finn, R. K., Halvorson, H. O., and Piert, E. L., 1950, Lactic Acid Fermentation Rate. Effect of Continuously Controlled pH., Ind. Eng. Chem., 42:1857.
7. Longworth, L. G., and MacInnes, D. A., 1935, Bacterial Growth with Automatic pH Control. A. An Apparatus. B. Some Tests on the Acid Production of Lactobacillus acidophilus, J. Bact., 29:595.
8. Vickers, A. E. J., Sugden, J. A., and Bell, R. A., 1932, An Apparatus for the Continuous Recording of pH, J. Soc. Chem. Ind., 51:545.
9. Whitnah, C. H., 1933, A Mechanically Operated Burette, Ind. Eng. Chem. Anal. Ed., 5:352.
10. Whittier, E. O., and Rogers, L. A., 1931, Continuous Fermentation in the Production of Lactic Acid, Ind. Eng. Chem., 23:532.
11. Longworth, L. G., and MacInnes, D. A., 1936, Bacterial Growth at Constant pH. Quantitative Studies on the Physiology of Lactobacillus acidophilus, J. Bact., 31:287.
12. Longworth, L. G., and MacInnes, D. A., 1936, Bacterial Growth at Constant pH. Apparent Oxidation-Reduction Potential, Acid Production, and Population Studies of Lactobacillus acidophilus under Anaerobic Conditions, J. Bact., 32:567.
13. Hickey, R. J., 1941, The Effect of Controlled pH upon the Production of Chemicals in Several Fermentations, Thesis, Iowa State College.

14. Fulmer, E. I., Underkofler, L. A., and Hickey, R. J., 1945, Glycerol Production, U. S. Patent 2,388,840.
15. Fulmer, E. I., Underkofler, L. A., and Hickey, R. J., 1947, Glycerol Production, U. S. Patent 2,416,745.
16. Underkofler, L. A., Fulmer, E. I., Lees, T. M., and Figard, P. H., 1951, Production of Glycerol by Fermentation. II. Fermentation of Disaccharides and Starch, Iowa State Coll. J. Sci., 26:135.
17. Underkofler, L. A., Fulmer, E. I., Hickey, R. J., and Lees, T. M., 1951, Production of Glycerol by Fermentation. I. Fermentation of Dextrose, Iowa State Coll. J. Sci., 26:111.
18. Leonard, R. H., Peterson, W. H., and Johnson, M. J., 1948, Lactic Acid from Fermentation of Sulfite Waste Liquor, Ind. Eng. Chem., 40:57.
19. Kepes, A., 1948, Appareillage et Technique D'Enregistrement et de Régulation Automatique du pH et de Potential D'Oxydo-Réduction dans les Milieux de Culture, Ann. Inst. Pasteur, 74:337.
20. Breeze, J. E., 1948, A Precision Electronic pH Control, Proc. Natl. Electronics Conf., 4:451.
21. Neish, A. C., and Ledingham, G. A., 1949, Production and Properties of 2,3-Butanediol. XXXII. 2,3-Butanediol Fermentations at Poised Hydrogen Ion Concentrations, Can. J. Res., B., 27:694.
22. Watson, R. W., Tamboline, F., and Harmsen, G. W., 1949, Production and Properties of 2,3-Butanediol. XXXIII. Automatic pH Control in the Dissimulation of Sucrase by Bacillus polymyxa, Can. J. Res., F., 27:457.
23. Brown, W. E., and Peterson, W. H., 1950, Factors Affecting Production of Penicillin in Semi-Pilot Plant Equipment, Ind. Eng. Chem., 42:1769.
24. Hosler, P., and Johnson, M. J., 1953, Penicillin from Chemically Defined Media, Ind. Eng. Chem., 45:871.
25. Kempe, L. L., Halvorson, H. O., and Piret, E. L., 1950, Effect of Continuously Controlled pH on Lactic Acid Fermentation, Ind. Eng. Chem., 42:1852.
26. Lakata, G. D., 1954, Apparatus for Controlled Submerged Fermentation, Applied Microbiol., 2:2.
27. Steinberg, M. P., and Ordal, Z. J., 1954, Effect of Fermentation Variables on Rate of Fat Formation by Rhodotorula gracilis, J. Agr. Food Chem., 2:873.
28. Lea, D. E., 1947, Actions of Radiations on Living Cells, The Macmillan Co., New York.

29. Risse, O., 1929, Einige Bemerkungen zum Mechanismus chemischen Röntgenreaktionen in wässrigen Lösungen, Strahlentherapie, 34:578.
30. Fricke, H., 1934, The Chemical-Physical Foundation for the Biological Effects of X-Rays, Cold Spring Harbor Symposia Quant. Biol., 2:241.
31. Dale, W. M., 1943, Effect of X-Rays on Aqueous Solutions of Biologically Active Compounds, Brit. J. Radiology, 16:171.
32. Dale, W. M., Meredith, W. J., and Tweedie, M. C. K., 1943, Mode of Action of Ionizing Radiations on Aqueous Solutions, Nature, 151:280.
33. Dale, W. M., 1943, Effects of X-Rays on Acetylcholine Solutions Showing Dilution and Protection Effect Phenomena Found for Enzymes, J. Physiol., 102:50.
34. Dale, W. M., 1942, The Effect of X-Rays on the Conjugated Protein D-Amino Acid Oxidase, Biochem. J., 36:80.
35. Dale, W. M., 1940, The Effect of X-Rays on Enzymes, Biochem. J., 34:1367.
36. Dale, W. M., 1952, The Indirect Action of Ionizing Radiations on Aqueous Solutions and its Dependence on the Chemical Structure of the Substrate, J. Cell. Comp. Physiol., 39, Suppl. 1:39.
37. Weiss, J., 1944, Radiochemistry of Aqueous Solutions, Nature, 153:748.
38. Dainton, F. S., 1948, On the Existence of Free Atoms and Radicals in Water and Aqueous Solutions Subjected to Ionizing Radiations, J. Phys. & Colloid. Chem., 52:490.
39. Dale, W. M., 1947, Action of Radiation on Aqueous Solutions: Experimental Work with Enzymes in Solution, Brit. J. Radiol., Suppl. 1:46.
40. Dale, W. M., Davies, J. V., and Meredith, J. W., 1949, Further Observations on Protection Effect in Radiation Chemistry, Brit. J. Cancer, 3:31.
41. Lea, D. E., Smith, K. M., Holmes, B., and Merkhham, R., 1944, Direct and Indirect Action of Radiation on Viruses and Enzymes, Parasitology, 36:110.
42. Luria, S. E., and Exner, F. M., 1941, The Inactivation of Bacteriophages by X-Rays—Influence of the Medium, Proc. Nat'l. Acad. Sci., 27:370.
43. Ephrati, E., 1948, The Mechanism of the Effect of X-Rays on Bacterial Toxins, Biochem. J., 42:383.
44. Forssberg, A., 1947, Mechanism of the Action of X-Rays on Enzymes in Water Solution, Nature, 159:308.

45. Goldblith, S., and Proctor, B. E., 1948, Effect of High-Voltage X-Rays and Cathode Rays on Vitamins (Niacin), Nucleonics, 3:32.
46. Lawrence, C. A. and Graikoski, J. T., in Progress Report 2, Utilization of the Gross Fission Products, Univ. of Mich., Eng. Res. Inst., Proj. M943, January, 1952, pg. 78.
47. Elliott, A. M., Brownell, L. E., and Gross, J. A., 1954, The Use of Tetrahymena to Evaluate the Effects of Gamma Radiation on Essential Nutrilites, J. Protozoology, 1:193.
48. Fisher, C. H., and Filachione, E. M., 1948, Lactic Acid-Versatile Intermediate for the Chemical Industry, U. S. Bureau Agr. and Ind. Chem., Circ. Ser. AIC-178 (May 1948).
49. Inskeep, G. C., Taylor, G. G., and Breitzke, W. C., 1952, Lactic Acid from Corn Sugar, Ind. Eng. Chem., 44:1955.
50. Considine, D. M., and Eckman, D. P., 1946, Advantages of High-Speed Electronic Recording in Industrial Process Measurements, Trans. Am. Inst. Chem. Eng., 42:707.
51. Clark, W. M., 1928, The Determination of Hydrogen Ions, 3rd Ed., The Williams and Wilkins Co., Baltimore.
52. MacLeod, R. A. and Snell, E. E., 1947, Some Mineral Requirements of the Lactic Acid Bacteria, J. Biol. Chem., 170:351.
53. Liggett, R. W., and Koffler, H., 1948, Corn Steep Liquor in Microbiology, Bact. Rev., 12:297.
54. Liggett, R. W., 1946, The Use of Corn Steeping Liquor in Microbiological Research, J. Bact., 51:597.
55. Peckham, G. T., Jr., 1944, The Commercial Manufacture of Lactic Acid, Chem. Eng. News, 22:440.
56. Jacobs, M. B., 1944, The Chemistry and Technology of Food and Food Products, 1st Ed., Interscience Publishers Inc., New York, pg. 454.
57. Hester, A. S., and Ladish, J. H., 1954, Malting, Ind. Eng. Chem., 46:1738.
58. Brownell, L. E., Meinke, W. W., Nehemias, J. V., and Coleman, E. W., 1953, Design and Use of Ten-Kilocurie Source of Gamma Radiation, Chem. Eng. Progress, 49:569.
59. Nehemias, J. V., Brownell, L. E., Meinke, W. W., and Coleman, E. W., 1954, Installation and Operation of Ten-Kilocurie Cobalt-60 Gamma-Radiation Source, Am. J. Physics, 22:88.
60. Lewis, J. G., Nehemias, J. V., Harmer, D. E., and Martin, J. J., 1954, Analysis of Radiation Fields of Two Gamma-Radiation Sources, Nucleonics, 12:40.

61. Weiss, J., 1952, Chemical Dosimetry Using Ferrous and Ceric Sulfates, Nucleonics, 10:28.
62. Shaffer, P. A., and Somogyi, M., 1933, Copper-Iodometric Reagents for Sugar Determination, J. Biol. Chem., 100:695.
63. Somogyi, M., 1952, Notes on Sugar Determination, J. Biol. Chem., 195:19.
64. Garner, R. L., Personal Communication.
65. Kolthoff, I. M., and Sandell, E. B., 1946, Textbook of Quantitative Inorganic Analysis, The MacMillan Co., New York, pg. 618.
66. Friedemann, T. E., and Graeser, J. B., 1933, The Determination of Lactic Acid, J. Biol. Chem., 100:291.
67. Friedemann, T. E., and Kendall, A. I., 1929, The Determination of Lactic Acid, J. Biol. Chem., 82:23.
68. Freeman, G. G., and Morrison, R. T., 1946, Determination of Some Products of Sugar and Molasses Fermentations, Analyst, 71:511.
69. Finn, R. K., 1949, The Rate of Formation of Lactic Acid by Fermentation at Controlled pH, Thesis, University of Minnesota.
70. Kempe, L. L., 1948, The Effect of Continuously Controlled pH on the Lactic Acid Fermentation, Thesis, University of Minnesota.
71. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., 1951, Manometric Techniques and Tissue Metabolism, 2nd Ed., Burgess Publishing Co., Minneapolis.
72. Doede, D. R., 1945, The Influence of pH upon the Growth-Factor Requirements of Bacteria, Yale J. Biol. Med., 17:595.
73. Simon, E. W., and Beevers, H., 1952, The Effect of pH on the Biological Activities of Weak Acids and Bases. I. The Most Usual Relationship Between pH and Activity, The New Phytologist, 51:163.
74. Halvorson, H. O., and Muedeking, M. R., 1947, A Comparison of Rates of Fermentation by Lactobacillus casei in Synthetic and Yeast Extract Media, J. Bact., 54:39.
75. Stiles, H. R., and Pruess, L. M., 1938, Nutrient Requirements of Lactobacillus delbrueckii in Lactic Acid Fermentation of Molasses, J. Bact., 36:149.
76. Wilson, P. W., and Knight, S. G., 1949, Experiments in Bacterial Physiology, Burgess Publishing Co., Minneapolis, pg. 46.
77. Hodgeman, C. D., 1947, Handbook of Chemistry and Physics, Chemical Rubber Publishing Co., Cleveland, pg. 1425.