THE UNIVERSITY OF MICHIGAN
INDUSTRY PROGRAM OF THE COLLEGE OF ENGINEERING

PROPOSED NEW PROCESS FOR THE MANUFACTURE OF LACTIC ACID

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INTRODUCTION

Lactic acid is among the oldest of the known organic compounds. Even though it has been manufactured for over one hundred years, the present day manufacturing process is not much different from the one used by Scheele in 1780, who obtained it from fermented milk.

The manufacturing process involves the fermentation of a fortified carbohydrate mash. The temperature is very closely controlled. An excess of calcium carbonate helps to maintain a very close pH control. The acid is neutralized as it is formed by the fermentative bacteria thus preventing the pH from reaching the low values which would inhibit the metabolic activity of the microorganism. The fermentation yields a raw product in the form of calcium lactate which is recovered from the fermented mash after the fermentation is completed.

Since lactic acid is sold in a number of different grades, the purification and recovery from the fermented liquor varies considerably. Usually the fermented liquor is treated with lime to pH 10 or pH 11. It is heated to boiling, killing the organisms present and coagulating the proteinaceous materials. This facilitates the filtration of the excess carbonate while keeping the calcium lactate in solution. The clarified calcium lactate solution is treated according to the desired final product.

The lactate is an inexpensive compound as it occurs in the diluted fermentation liquors. The relatively high cost of the more refined grades can be largely attributed to the difficulties encountered during the subsequent purification steps. The fermented liquor

contains very troublesome impurities such as dextrins, proteins, inorganic salts and unfermented sugars.

The acid has a very low vapor pressure and a tendency to undergo auto-esterification, which makes it difficult to purify by distillation.

Very careful considerations of all the difficulties encountered in producing lactic acid by the present day process has led to the contentions that it might be feasible to develop a basicly new process for its manufacture.

STATEMENT OF THE RESEARCH PROBLEM

The research problem treated in this thesis deals with the quantitative study of the feasibility of developing a basicly new process for the manufacture of lactic acid.

The process under consideration will consist of the following steps which are based on the qualitative results of exploratory experiments performed during the Fall of 1954.

1. Fermentation: The fermentation is to be performed in such a way as to obtain lactic acid in the acid form rather than as a salt, at the end of the fermentation. The effect of lactate salts upon the extent of ionization of lactic acid is utilized in place of the usual neutralization with calcium carbonate as the acid is formed.

2. Extraction: The acid lactate produced in the fermentation will be recovered from the fermented liquor by means of a selective solvent.

3. Distillation: The solvent used in the liquid-liquid extraction will be distilled from the lactate-rich solvent phase in order to obtain the lactic acid.

4. Recirculation: The extracted liquor will be recycled back to the fermentor.

Each of the above steps will be studied in a quantitative fashion during the course of the experimental program.

LITERATURE SEARCH

A very extensive literature search was performed on the general topic of lactic acid. However, many details were kept in mind when investigating its chemical and physical properties, methods of manufacture and purification schemes. What follows will set forth the basic information on lactic acid.

A. General

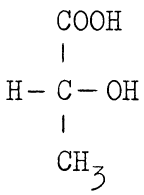
Lactic acid or α-hydroxypropionic acid is an interesting and important chemical (3) for several reasons. In addition to being one of the earliest known organic acids (5, 10) it is perhaps the most widely distributed one in nature (40). It is one of the most prevalent acids of the soil and the primary acid constituent of sour milk (18), from whence it gets its name. It is also a normal constituent of blood and of the muscle tissue of animals (40). It is present in many fermented substances, such as: sour milk, sauerkraut, fermented mash or beer and in many food products such as breads (1, 40). It is the simplest hydroxy acid having an asymmetric carbon atom. It is a very important chemical in theoretical chemistry, biological processes and in various industrial fields (3).

The earlier studies of lactic acid were primarily concerned with the physiological or stereochemistry phenomenon and related fields (41,. . ., 48). However, most of the recent work reflects a growing interest in the industrial applications of the acid, as an intermediate for various industrially valuable products, such as chemical intermediates, solvents, plasticizers, resins and elastomers (3, 49).

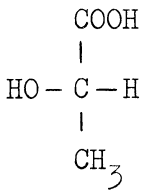
Lactic acid was first prepared by Scheele in 1780, who according to LaGrange (2), allowed milk to ferment for three weeks. The fermented liquor was deproteinized and treated with calcium hydroxide to precipitate the phosphates. The calcium lactate-rich filtrate was treated with oxalic acid, thus regenerating the acid lactate and precipitating the calcium as the oxalate which was filtered off. The filtrate was concentrated and extracted with alcohol. The acid lactate obtained after evaporation of the alcohol was a thick syrup which could not be distilled or crystallized.

Having an asymmetric carbon atom, lactic acid is capable of existing as the d-, l- and dl- forms, all of which occur freely in nature, either mixed or in the pure state. However, the commerical technology of the acid makes no distinction. Commercial lactic acid is usually the racemic mixture (1, 15, 40).

Much confusion has existed until recently regarding the nomenclature used to designate the isomeric forms (15, 17, 40). The form of the acid commonly known as sarcolactic acid which occurs in blood has a (+) rotation but an "l" configuration. This isomer is properly designated by modern nomenclature as L (+) - lactic acid (II). The optical isomer which has a (-) rotation but a "d" configuration is designated as D (-) - lactic acid (I).



(I) D(-) - Lactic acid



(II) L(+) - Lactic acid

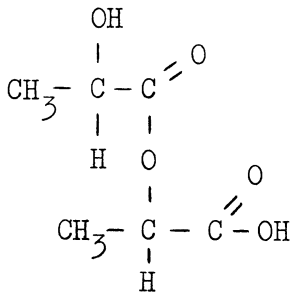
The salts of the dextrorotatory acid, are levorotatory and those of the levorotatory acid are dextrorotatory. Each of the optically active acids can be converted by heating into the corresponding optically inactive racemic form which contains equal portions of the dextro- and levorotatory forms (1, 40).

Due to the low optical rotatory power of the free acids, the rotation of the acids or of its simple salts cannot be considered a valuable criterion to establish either the optical forms of the acids or the percentage composition in the case of mixtures (15, 17, 44). The resolution of a mixture can be accomplished with difficulty by means of the zinc salts (15).

The benzimidazole derivatives of lactic acid have greater rotatory power, so that either form of the lactic acid or the percentage composition of a mixture can be identified readily by the benzimidazole derivative (7, 20).

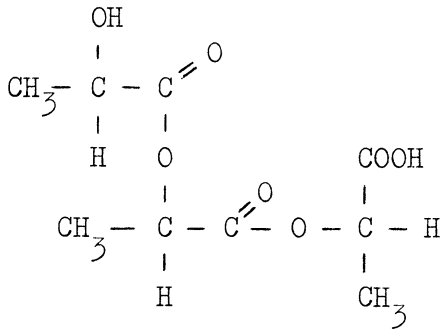
Even if the concentration of an aqueous solution of lactic acid is conducted at relatively low temperatures and in the absence of esterification catalysts (6, 19, 11), the removal of water is accompanied by the auto-esterification of the acid.

According to Thorpe (18), the auto-esterification of the acid can yield four different anhydrides which are differentiated from one another by the conditions to which the acid is subjected. Lactyl-lactic acid:



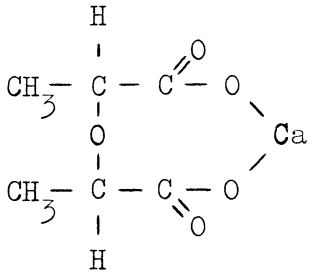
is obtained by heating lactic acid at 130-140°C (8, 12). It is also formed to an appreciable extent by merely keeping lactic acid in a desiccator at ordinary temperatures (13). This anhydride is a pale yellow, low-melting, amorphous mass which is only sparingly soluble in water. Lactic acid is regenerated upon long boiling with water, or by the action of alkalies (1, 18).

Lactyl-lactyl-lactic acid, commonly known as "trilactic acid",

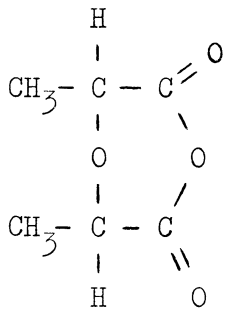


is formed when lactic acid is heated for several hours at 90-100°C/25 mm Hg. The "trilactic acid" forms needles having a melting point of 39°C and a boiling point of 235-240°C/20 mm Hg (14).

When calcium lactate is slowly heated to 180°C it forms the calcium salts of dilactylic acid.

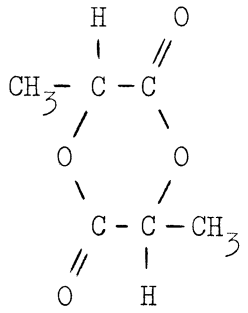


The free acid is very soluble in water and is easily crystallized from benzene. The leaflets melt at 106°C (18). Vacuum distillation of a water solution yields the anhydride,



having a boiling point of 110°C/20mm Hg (4).

Slow vacuum distillation of lactic acid at 140°C/10 mm Hg yields the lactide (16)



which crystallizes in colorless tablets having a melting point of 120°C and a boiling point of 255°C/760 mm Hg or 138°C/12 mm Hg (18). It is only slightly soluble in cold water and alcohol but very soluble in acetone (1, 16, 18). The lactide can be prepared also by the vacuum distillation of sodium α-bromopropionate (9). It is slowly hydrolyzed to lactic acid by prolonged heating with water (1, 16).

These several components, including water, occur in various proportions in aqueous lactic acid. The extreme limits are pure water and the completely polymerized lactic acid, $(- O C H (CH_3) C O -)_x$. Figure 1 shows the composition of aqueous lactic acid at equilibrium and at progressive stages of dehydration. The data were taken from Bezzi (6) and Watson (11).

For example let us say that we have a lactic acid-water solution containing ten percent water. The vertical axis indicates a

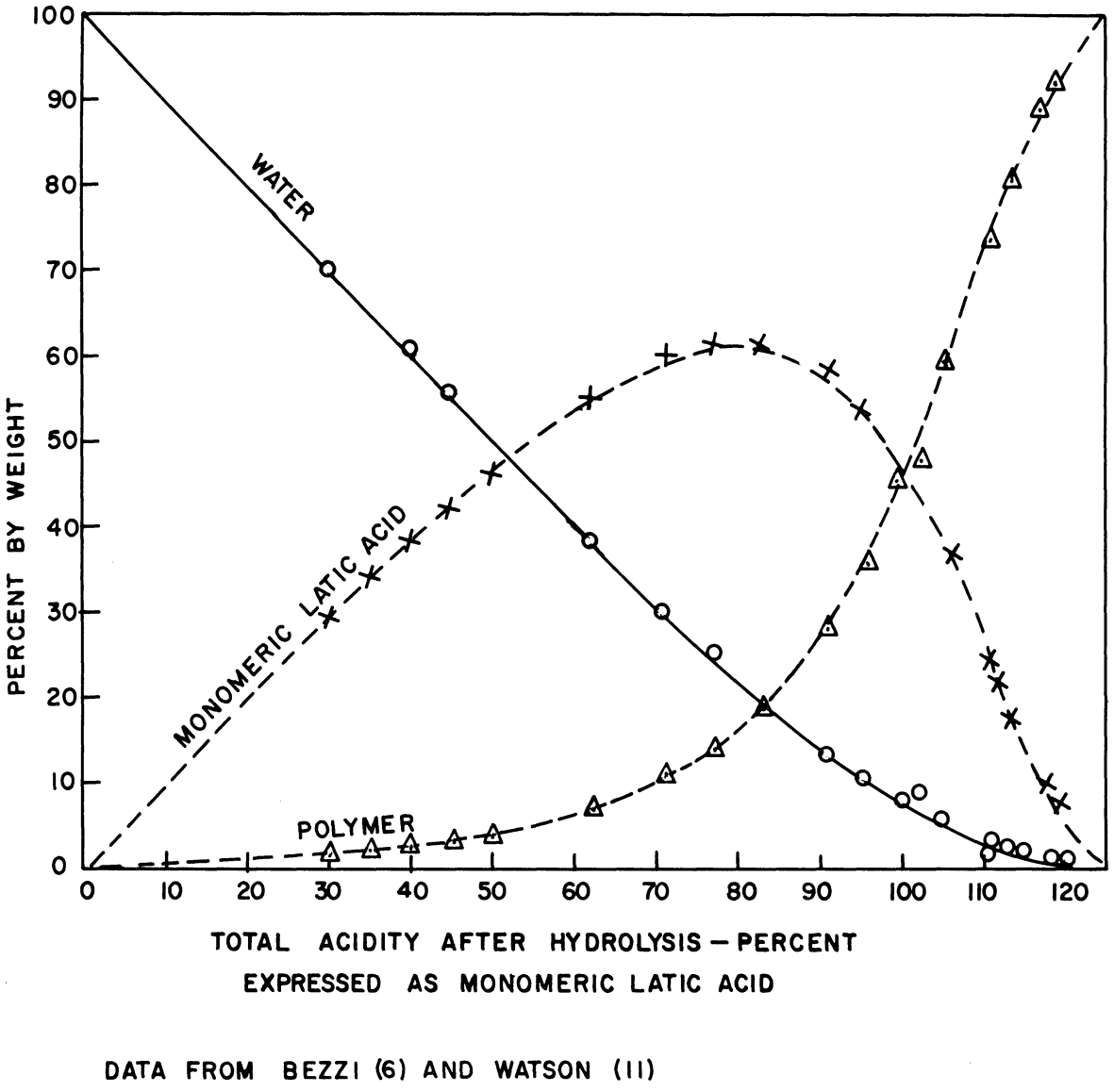


Figure 1. Compositions of Aqueous Lactic Acid Solutions and Dehydrated Lactic Acid

concentration of fifty five percent by weight as monomeric lactic acid in equilibrium with a concentration of thirty five percent by weight of the completely polymerized lactic acid. If the polymerized fraction is completely hydrolyzed, the acidity, expressed as monomeric lactic acid, would be approximately ninety six percent which can be read on the horizontal axis.

Lactic acid undergoes esterification readily with many alcohols. Numerous lactic acid esters have been prepared by acid-catalyzed reactions and a number of them have been described in the literature (50). Previous to 1940, the yields reported were frequently low probably due to the fact that the esterification is complicated by the formation of auto-esterification products such as polylactic acid (3, 50, 58). The formation of the polylactic ester can be minimized by using a large excess of the alcohol during the esterification with the subsequent rapid removal of the water and the excess alcohol from the ester (50). It is also necessary to neutralize the esterification acid catalyst with a base, such as sodium acetate, after the esterification is completed, in order to prevent the self-alcoholosis of the ester during the isolation by distillation. Extensive research has been done on the esterification reactions and the conditions have been determined at which high yields can be obtained (51, . . ., 57). Smith and Claborn (50) discussed the method of preparation of many of the low-boiling esters soluble in water, esters insoluble in water and the high-boiling water-soluble esters. The esters are suitable for a number of important potential uses in laquers, varnishes, inks, stencils, pastes, and organosols (3).

Lactic acid is a colorless to slightly yellowish sirupy liquid with a specific gravity of 1.294 at 25°C. It is completely soluble in water, ethyl alcohol, and ether but insoluble in chloroform. As shown in Table I (17) it is fairly hygroscopic in solutions of high concentrations (17, 1), and with ferric chloride it gives the characteristic yellow-green color typical of α-hydroxy acids (1).

Because of the ease with which lactic acid undergoes autoesterification when heated, it has been difficult to determine the boiling point at most pressures and impossible at atmospheric pressure. As calculated by the method of Kinney (59, 60), the boiling point of the racemic mixture at atmospheric pressure is approximately 190°C (105). Boiling point values of 119°C/12 mm Hg and 82-85°C/1mm Hg has been reported by Borsook, et al. (17). Fischer and Filachione (58) estimated the boiling point from the boiling points of various lactic esters. They also related the boiling points of various acids and their esters. Their results indicate also a boiling point of approximately 190°C/760 mm Hg.

B. Chemical Production of Lactic Acid

Lactic acid can be obtained from a number of chemical reactions that have been studied from time to time. While some of the reactions show some promise from the industrial point of view, most of them must be considered only as classical methods of preparing the acid.

The acid has been obtained by the following methods:

1. Alkaline hydrolysis of α-halogenated propionic acid (122,. . ., 125).

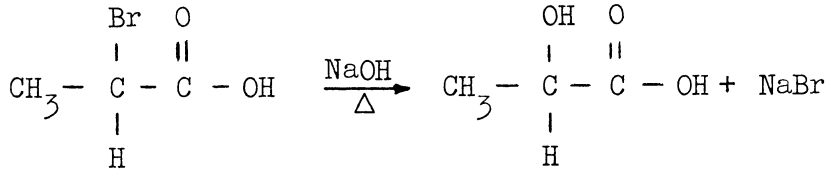
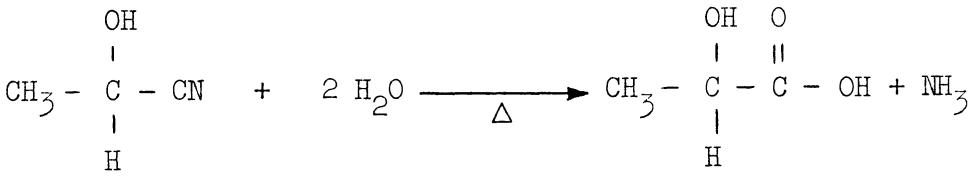


TABLE I. PROPERTIES OF OPTICALLY ACTIVE LACTIC ACIDS*

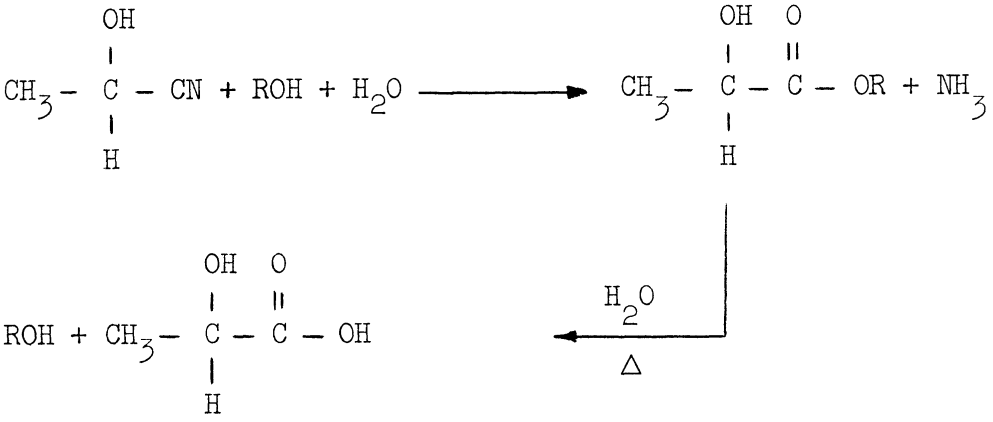
	Melting Point °C	Water absorbed from air at room temp.	Dissociation constant at 25°C pK
D(-)Lactic acid	52.8	0 in 4 hrs; 2% in 20 hrs.	3.83 ± 0.01
L(+)Lactic acid	52.8	1% in 4 hrs; 3% in 20 hrs.	3.79 ± 0.01
DL Lactic acid	16.8	3% in 3 hrs; 10% in 15 hrs.	3.81 ± 0.01

* Data from Borsook, et al. (17).

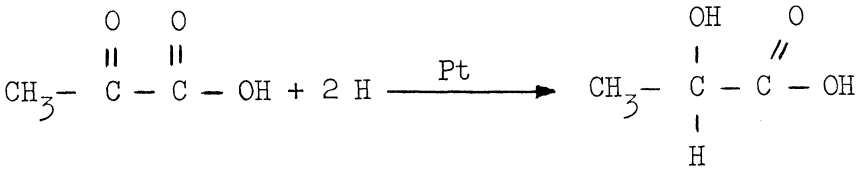
2. Acid hydrolysis of acetaldehyde cyanhydrin (lactonitrile) (126, 127, 129).



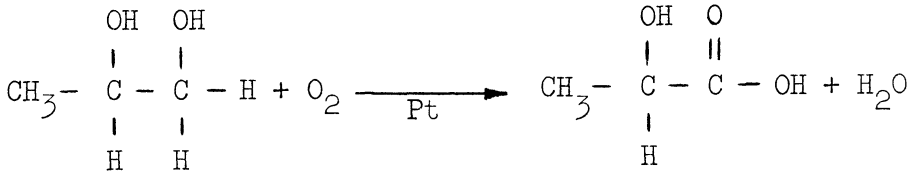
or by the esterification of the lactonitrile into the ester with the subsequent hydrolysis of the ester (128).



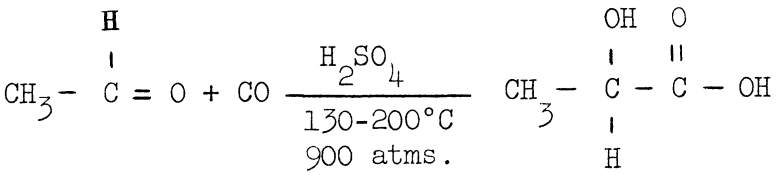
3. Reduction of pyruvic acid (130, . . ., 132).



4. Catalytic oxidation of 1,2-propylene glycol (133,134).



5. Acetaldehyde and carbon monoxide (118).



6. Alkaline degradation of carbohydrates.

Evans (135) has reviewed sixty one references on the less known aspects of carbohydrate chemistry with special emphasis on the alkaline conversion of hexose sugars into lactic acid. Montgomery (136) has also reviewed the chemical preparation of lactic acid from sugars and reported the detailed results of all the experimental work done on the subject prior to 1949. Montgomery and Ronca (115) performed a detailed investigation on the production of lactic acid by the alkaline degradation of carbohydrates, especially those present in black strap molasses. Although the yields were found to be lower than those of fermentation, the reaction time needed to be only a few minutes long and could be carried out at 40 percent sugar concentrations. The process could be run continuously.

C. Present Day Fermentation Process

1. General

The commercial production of lactic acid in the United States is performed through the fermentation of cheap raw materials. The ones most commonly used are molasses, starch hydrolyzates, dextrose or whey (1, 3, 15, 18, 40, 58, 61, 62, 64, 66, ..., 73, 75).

The fermentation procedure in general consists of the fermentation of a carbohydrate into which suitable mineral and proteinaceous nutrients have been incorporated. A very close control of the temperature is maintained and an excess of calcium carbonate is always present in order to provide a close control of the pH. The calcium carbonate neutralizes the acid as it is formed thus preventing the pH from reaching low values which would inhibit the fermentation. The carbohydrate concentration is usually adjusted to about 12 percent concentration so that when the fermentation is completed the liquor will contain from

12-15 percent calcium lactate (1). Higher sugar concentration will result in calcium lactate crystallizing out at the fermentation temperature thus making the fermenting liquor unduly heavy and difficult to handle. The fermentation progresses very rapidly during the first forty-eight hours during which time the sugar concentration frequently decreases as much as 3 percent each 24 hours. The fermentation rates decrease as the fermentation reaches completion. When most of the sugar has been fermented it may disappear as slowly as 0.1-0.2 percent per day. Although the fermentation time will vary from batch to batch it usually ranges from 5 to 10 days.

Due to the difficulties encountered with the residual carbohydrates (15) during the processing of the acid liquor it is very important to carry the fermentation as near to completion as possible. The residual carbohydrates must be reduced to less than 0.1 percent of the original concentration.

After the fermentation is finished, the fermented liquor is treated with lime to pH 10 or pH 11. The liquor is then heated to boiling to kill the organisms present. The proteinaceous material coagulates, thus facilitating the filtering of the excess calcium carbonate and cellular mass. Any remaining unfermented sugar is degraded by the lime during the boiling of the liquor.

Generally it has been preferred to use thermophilic bacteria of the Lactobacillus delbrueckii type because they exhibit their optimum temperature on the range from 45° to 50°C (1, 15, 72). Performing the fermentation at this high a temperature usually eliminates most contamination problems. It also permits the use of a fermenting liquor that has been pasteurized, as opposed to the sterilized media usually

required for mesophilic bacteria. This represents a considerable saving in the amount of steam used and in the equipment required.

According to the following overall reaction,

$$C_6H_{12}O_6 \longrightarrow 2\ CH_3 - CHOH - COOH$$

$$180 \longrightarrow 2\ (90)$$

the theoretical yield of lactic acid is 100 percent of the weight of fermented hexose sugar. However, this yield is never obtained in actual practice. Presumably the organism uses a portion of the carbohydrate in its metabolism, so that fermentation yields of 94 to 96 percent are normal. Further losses will occur during the subsequent processing and purification of the product. The final yield will vary from plant to plant, but yields of 85 percent are normal (1, 3, 15, 18, 40, 58).

2. Grades

Since lactic acid is sold in a number of different grades, as shown in Table II (139), the purification and recovery of the acid from the fermented liquor will vary considerably. As examples of these variations we can mention the following: (1, 3, 15, 38, 40, 61, 66, 67, 68, 69, 72).

The crude acid or technical grade which is of a rather indefinite color is sold in 22, 44, 50, 66, 80 percent and other concentrations all depending on the customer's specifications. Since it does not require a high degree of purity, it can be obtained by the direct acidification of the fermented liquor or by the direct decomposition of the raw calcium lactate with sulfuric acid. The calcium sulfate is filtered off and the acid liquor is concentrated to the desired level. Most of the calcium sulfate is removed by filtration

at about 30-35 percent concentration. Such a crude technical acid will contain many of the impurities which are present in the starting material, thus the quality of the subtrate will determine the quality of the raw acid obtained from it.

The edible grade lactic acid which is a light, straw-colored product and is customarily sold in 55 to 80 percent concentrations, may be made by two different procedures. Whether one chooses to refine the substrate or the final product will be dictated by the economics of each. One method uses a fermentation mash composed of very refined sugar, a minimum of nutrient material and a relatively pure calcium carbonate. After the fermentation is finished the liquor is acidified with sulfuric acid and the calcium sulfate is removed by filtration. The acid is concentrated and decolorized with vegetable carbon. The iron and copper impurities may be removed as the cyanides. The second method utilizes an inexpensive carbohydrate substrate. The resulting calcium lactate liquor is clarified by filtration and concentrated to about 25 percent. The calcium lactate precipitates upon cooling and is separated by filtration or centrifugation. It is redissolved in water and acidified with sulfuric acid. From here on both processes are identical.

The plastic and U. S. P. grades of lactic acid are usually made from very pure substrates. The calcium lactate obtained from the fermentation is purified by recrystallization, redissolved and subsequently acidified with sulfuric acid. The acid liquor is decolorized with animal charcoal. The lactic acid obtained by this method usually meets the specifications of the edible, U. S. P. and plastic grade suitable for colored phenolic resins. However, the chemically pure

TABLE II. PRICES OF VARIOUS GRADES OF LACTIC ACID IN OCTOBER, 1956*

Grade	Concentration Weight Percent	Cents per Pound As Sold	Cents per Pound 100% Basis
Technical	22	6.3	28.7
Edible	50	22.5	45.0
	80	37.5	46.8
Plastic	50	23.5	47.0
	80	37.5	46.8
U. S. P.	85	88.0	103.5

* Data from: Oil, Paint and Drug Reporter, October 29, 1956 (139).

lactic acid is made by converting the calcium lactate into the zinc salt by the use of zinc carbonate or zinc sulfate. The zinc lactate is recrystallized and dissolved in water. The zinc is precipitated as the zinc sulfide and the solution is decolorized with animal charcoal. The zinc salt is best suited for this operation since it crystallizes better than any other lactate salt.

As a rule each consumer sets up his own specifications for the acid and these will depend upon its uses. In the case of the edible grade lactic acid there are no specifications except the general requirement for food; that it shall not contain harmful ingredients in sufficient quantity to be detrimental to the health. He may also specify color, odor and flavor. In the case of lactic acid to be used in the manufacture of transparent cast phenolic resins, the specifications are very rigid with respect to: acidity > 50 percent, chlorides (as chlorine) < 5 ppm, sulfates (as $SO_4^{=}$) < 50 ppm, ash < 0.05 percent, iron, not more than a trace (38). The product must be water clear. The iron impurities react with phenol to give colored compounds. The other salts that could be present are insoluble in the resin and they cause a haze or opalescence in the finished product.

Since the specifications for the acid to be used in plastics are so rigid, considerable difficulty has been encountered in its production at prices that will permit larger consumption. However, a number of new processes aiming at the cheaper production of the higher grades have been developed during the past ten or fifteen years. To the author's knowledge they have not been used industrially as of yet; however, they will be discussed later in the purification section.

3. Raw Materials

A wide variety of cheap raw materials may be used for the industrial production of lactic acid by the processes involving the fermentation of carbohydrates. The difficulties encountered during the purification of the fermented liquors restrict somewhat the number and type of raw materials actually used. Industry has generally assumed that it is more advantageous to perform the fermentation step with a "cleaner" carbohydrate than to attempt much purification of the fermented liquor (1, 15). It has been a matter of economics whether one chooses to refine the substrate before the fermentation or the fermentated liquor with the subsequent purification of the lactic acid. Dextrose has probably been more widely used than any other substrate for lactic acid production (1, 15). A staff-industry report (68) has recently been published which reviews in considerable detail all phases of the production of lactic acid from dextrose.

Smith and Claborn (6) have considered whey to be a partially important raw material for lactic acid fermentation. It seems that the cost of collecting the whey and general processing problems have prevented this source from assuming greater importance (1). Burton (67) has described the operation of a lactic acid plant using whey. Whittier and Rogers (73) developed a laboratory method for the continuous fermentation of whey which later was adapted to commercial operation.

The possibility of using pentose sugars like xylose and arabinose has been studied (83, 84) using the pentose fermenting organism Lactobacillus pentoaceticus.

The utilization of sulfite wate liquor has been studied by Fries (85) and Peterson (86) as a method of eliminating stream pollution problems where paper pulp mills are located.

Peterson (87) studied the fermentation of sugar cane black strap molasses and his results were very favorable. Molasses (93) are not used extensively at the present time due to the large percentage of impurities they contain. No reference has been made to their purification by the method (88) which is extensively used in the production of yeast. Other raw materials which have been mentioned from time to time for the manufacture of lactic acid include Jerusalem artichokes (90), potatoes (94), beet juice and cassava roots (1).

The spent beer stillage from the alcoholic fermentation and the steep water from the corn wet-milling process contain considerable quantities of lactic acid calculated on a dry substance basis. However, the author does not know of any suitable recovery process, although it has been attempted by Laszloffy (95).

Most of the carbohydrate media used for fermentations must be supplemented with inorganic salts (92) and nitrogenous substances in order to stimulate the growth of the bacteria. It has been proved (92, 91) that the precise requirements will vary with the organism used in the fermentation. For the most part they seem to require the vitamins essential to animals (89, 91). Thus, a cheap source of these growth promoting substances must be available. It has been shown that malt sprouts, corn steep liquor, and thin grain residue serve as suitable accessory nutrient sources (87, 89, 91, 1).

4. Fermentative Bacteria

As stated by Peckham (15) many bacteria are capable of producing lactic acid especially those of the genus Lactobacillus. Some of the molds (70, 96) are also adaptable for the fermentation. Frequently other products are also produced in considerable amounts, especially acetic acid and ethyl alcohol (72). It has become customary to classify the lactic acid producing organisms (1, 72, 97) into those which produce substantially only lactic acid from hexose sugars and those which produce other acids or alcoholic compounds in addition to lactic acid. These two groups of bacteria are referred to as homofermentative and heterofermentative respectively. The homofermentative organisms produce lactic acid in yields ranging from 95-100 percent based on the sugar utilized.

The homofermentative organisms are most commonly used by industry and are generally long, slender, Gram-positive rods, nomotile, nonsporulating and produce only little gas which is chiefly carbon dioxide (1, 15). Strains of Lactobacillus delbrueckii are probably the most widely used of the homofermentative organisms. This organism is capable of quantitatively fermenting maltose, dextrose, and sucrose when properly adapted (1, 72).

When using whey as the substrate the organism most commonly used is Lactobacillus bulgaricus (61, 67, 72, 73, 75) since it is capable of fermenting lactose while Lactobacillus delbrueckii is not.

Lactobacillus pentoaceticus has been used when fermenting the pentoses in sulfite waste liquor (85, 86).

The production of lactic acid by mold fermentation has been investigated at length by several workers. Yields of 70-75 percent of

dextro lactic acid have been reported (70, 96) using Rhizopus Oryzae in submerged cultures.

5. Conditions

The extent, rate and yield of the fermentation is very dependent upon the conditions at which it is performed. Among these conditions we can mention the following:

Temperature of the fermentation: The lactic acid fermentation is performed at comparatively high temperatures. The optimum temperature for fermentations with commercial types of lactic acid organisms is about 45°C and this temperature must be carefully maintained (1, 15, 66, 67, 68, 72, 73). A temperature this high is outside the growth range for most contaminating organisms, thus contamination is not a very serious problem. However, if the temperature drops somewhat during fermentation, conditions may become very favorable for the butyric acid fermentation (72).

Hydrogen ion concentration - pH: It has been shown that the fermentation proceeds best when the pH is on the acid side of neutrality from a pH 5.5 to pH 6.0. In industry this pH is maintained by adding an excess of calcium carbonate to the fermenting liquor and agitating in order to keep the neutralizing agent in suspension. Extensive laboratory work has been performed by a number of investigators (79, 99, . . ., 102).

Kempe, et al. (102, 103) and Gillies (104) showed that the homofermentative characteristic of Lactobacillus delbrueckii is accentuated by adjusting the pH to lower values. The fermentation rates are somewhat lower at those pH values.

Substrate concentration: The maximum sugar concentration is normally limited by the solubility of the resulting calcium lactate, which is approximately 15 percent, at the temperature of the fermentation. The fermentation rate is not affected by the sugar concentration (102, 103, 104).

Oxygen requirements: The organisms used in the industrial fermentations are usually microaerophilic or anaerobic in nature.

D. Purification

1. General

The lactic acid industry is characterized by the fact that the acid is sold in several different grades and at prices noticeably higher than the cost of the sugars used as the raw carbohydrates in its fermentative production. The fermentable sugars in molasses, whey, dextrose and starch hydrolyzates may be obtained at prices ranging from 1 to 7 cents (1, 3) per pound. Since the yield of lactic acid ranges from 85-95 percent by weight of sugar utilized (15), the difference in price for the crude and U. S. P. grades of the acid becomes more noticeable. The data given in Table II (139) shows this fact quite markedly. According to Groggins (3) the calculated material cost of manufacturing the acid from sugar can be approximately represented by the following equation:

Materials cost of lactic acid = 1.136 (cost of sugar) + 1.77.

This equation relates the cost, in cents per pound of acid, of the raw materials only. On this basis, when fermentable sugars are available at 1 or 5 cents per pound, the calculated material cost of lactic acid would be 2.91 and 7.45 cents per pound. This shows the fact that lactic acid, as it occurs in the dilute fermentation liquors, is an inexpensive

compound, and that the relatively high cost of the more refined grades can be largely attributed to the difficulties encountered in the purification. This step of the manufacturing process of fermentating lactic acid is difficult largely because of the low vapor pressure of the acid and its tendency to undergo auto-esterification as was previously discussed. The acid is infinitely soluble in water (3). Also, the dilute fermentation liquors contain very troublesome impurities (1, 3, 15, 18, 27, 29, 30, 38, 40, 50, 55, 68, 72, 74) such as dextrins, proteins, inorganic salts and unfermented sugars. Researchers came to realize these facts at the turn of the century and a number of purification schemes have been proposed during the past fifty years.

2. Proposed Purification Schemes

Most of the following discussion is based upon information from patented processes, thus a large portion of it is meager in details. However, the author has expanded on the available information whenever the proposed processes have been so obvious that the expansion could be warranted by common knowledge about the acid at the present time.

Perhaps the oldest of the purification schemes is that of steam distillation under high vacuum as proposed by Macallum (35) and Haag (28, 31, 37). The purification starts with a crude acid lactate solution which has been obtained either from a fermentation or from the acid hydrolysis of lactonitrile. The solution is steam distilled under vacuum at a temperature of 60-120°F. The distillate is treated with an oxidizing agent (28, 31) such as nitric acid or hydrogen peroxide to decompose the color and odor imparting impurities that have distilled over. A second steam distillation removes all traces of the

carbonizable organic impurities. The method is rendered inefficient due to the relatively low recovery of high quality acid (1, 15, 38). A very large portion of the original acid is decomposed and the residue remaining in the still is a black tarry mass which is composed largely of the anhydrides and higher poly-lactic acids. It has a very limited recovery value. The yield and the quality of the purified acid can be controlled by the quality of the acid fed to the still. The distillate will invariably show the presence of carbonizable organic impurities if the feed has been obtained from a fermentation. This is most noticeable in cases when a portion of the carbohydrate material remains after the fermentation is completed.

Lawrie (36) purified the acid by quickly heating under vacuum the impure solution as a thin film on a hot surface kept at 230-315°F. He claimed that he was able to obtain almost instantaneous evaporation of the acid. No mention is made of whether a black tarry mass is deposited on the hot surface. Furthermore no information is given on whether it is necessary to subject the condensate to further purification schemes which could very well be the case since some of the acid must evaporate as the anhydride and lactyl-lactic acid forms.

Noerdlinger, et al. (32, 33, 34) utilized a gas humidification scheme in order to prepare pure lactic acid by distillation of the technical grade product. The crude acid lactate solution was first concentrated by absorbing the water with a stream of gas. Afterwards, the pure acid was removed by the continued passage of the current of gas. A gas temperature not to exceed 120-130°C was recommended in order to prevent the decomposition of the acid. Air or any other gas

which does not react chemically with the acid may be used. The patents do not mention the purity and quality of the product. If it is remembered that the lactyl-lactic acid is formed at a temperature of 90°C and boils at 130°C it can be concluded that the product will consist largely of the anhydrides.

Landau (27, 29, 30) proposed the following scheme. During the preparation of pure lactic acid by the extraction of the technical grade acid with an alcohol, the technical grade is first decolorized with charcoal or kielsguhr and concentrated as highly as possible. The concentrate is treated next with ethyl alcohol to precipitate the sugars and dextrins remaining from the fermentation. The concentrate is then extracted with the solvent. After distillation of the solvent the acid is obtained as the residue. The purity and quality of the acid obtained will depend upon the temperature at which the distillation is performed.

Klapproth (26, 39) extracted the acid with a solvent such as ether or a high alcohol in the presence of sulfuric acid and sodium sulfate. He also recommends any other strong acid and the salt of the acid. This fact indicates that he started with a lactic acid salt which was regenerated by sulfuric acid or "any other strong acid". The extract was washed with a concentrated solution of a neutral lactate such as that of sodium, calcium or barium in order to neutralize any excess sulfuric acid. The purity and the quality of the product is dependent also in this scheme upon the temperature at which the solvent is distilled.

Wülfing (25) purified fermentation lactic acid from butyric, caproic and other of the higher fatty acids. The scheme consisted of

treating the acid lactate solution with a volatile solvent such as carbon disulfide, methyl trichloride, carbon tetrachloride and ethylene dichloride in which the lactic acid is insoluble.

Jeneman (24) extracted the crude acid continuously in a counter current system using isopropyl ether as the solvent. The acid is recovered from the solvent phase by counter current washing with water. The aqueous solution is concentrated by evaporation. The product obtained by this method is almost free from ash, however it might contain traces of impurities resulting from the carbohydrates used in the fermentation. The acid must be refined further with activated charcoal.

Olson (23) proposed to utilize the solubility properties of the lactide and the anhydride. The crude lactate was precipitated and separated as the calcium salt. After regeneration of the acid lactate with an aqueous solution of sulfuric acid, the mixture of $CaSO_4$ and lactic acid was dried by evaporation at about 100°C. The dried mixture was heated to 130°C in order to convert the acid into the lactide and lactic anhydride. The soluble impurities were washed out with water. The purified mixture was treated with superheated steam at 230-240°F in order to hydrolize the product back to lactic acid.

Schatzkes (21) and Hamburger (22) recommended the following purification scheme. The impure lactic acid solution is treated with a magnesium compound, such as the carbonate, to form magnesium lactate. The magnesium lactate is separated and suspended in acetone, ether or any volatile solvent in which the lactic acid is completely soluble. However, the magnesium salt of the mineral acid used to regenerate the acid lactate should not be soluble in the solvent. The magnesium lactate-solvent suspension is next treated with sulfuric acid. The

magnesium sulfate separates and the solvent-lactic acid solution is evaporated.

A number of investigators (19, 38, 50, 51, 53, 55, 82, 106,. . ., 114) have proposed to use the lactic acid esters as the pathway toward the plastic grade acid. The method and the chemical reactions were originally proposed by Hillringhaus and Heilman (106) and by Byk (110). Several recent modifications have been proposed. In general the method consists of preparing the low boiling esters of lactic acid, methyl- or ethyl-, distilling them free from impurities, and hydrolyzing the esters by boiling in dilute water solutions. The alcohol liberated is recycled back to the esterifying pot.

As early as 1939, Smith and Claborn (38) were able to prepare pure water-clear acid. They started with "dry" calcium lactate which was dissolved in a large excess of methyl alcohol. The solution was acidified with sulfuric acid and the calcium sulfate was removed by filtration. The excess methyl alcohol was distilled at atmospheric pressure after the esterification was completed. The water and methyl lactate were distilled under vacuum. After diluting the mixture of methyl-lactate and water it was boiled to hydrolyze the ester. The authors reported a yield of 85 percent pure acid. At a later date the same authors published a report (50) describing in detail the methods used for the preparation of the lactic acid esters. They also published a rather complete list of the esters known to that time (50).

Wenker (108) patented a process by which he was able to esterify the 70-85 percent acid. He used about 1.5 moles of methanol, ethanol or isopropanol for every mole of lactic acid. Sulfuric acid was used as the esterifying catalyst.

Rehberg (51) developed a one step method for transforming lactic acid into methylacetoxypropionate by the reaction of lactic acid with methyl acetate. Both methyl lactate and methylacetoxypropionate were formed in 63.6 percent and 28.4 percent yield respectively.

Schopmeyer and Arnold (107) patented a process for the continuous esterification, distillation and hydrolysis for the production of lactic acid. They used methyl alcohol and sulfuric acid.

Filachione, et al. (53, 55) studied various details of the process especially the ratio of alcohol to acid, catalyst concentration, temperature and pressure. They used the alcohol in the vapor phase. Their continuous esterifying reactor consisted of a small glass column packed with Berl Saddles which was kept at 100°C or higher temperatures. The lactic acid-H_2SO_4 was fed at the top in the liquid form and the methanol vapors at the bottom. They studied the reaction using both water solutions of lactic acid and the molten polylactic acid which was prepared by vacuum evaporation of the dilute solution.

Dietz (82) proposed a modification to the process which consisted of extracting the ester directly from the dilute solution and subsequent hydrolysis.

Troupe and Kobe (109, 11, 113) studied in detail the kinetics of the esterification reaction and were able to correlate empirically all of their data. They also proposed methods of analysis for the lactic acid-lactate esters systems.

Horsley (112) reported azeotropes of methyl and ethyl lactate.

Underkofler and Hickey (1) are of the opinion that the esterification process of purifying lactic acid is probably the most

practicable of all. The process looked so promising that the Sugar Research Foundation contracted the services of Needle and Aries (114) to perform a complete economic analysis of this process.

The author does not know of any industrial application of the process as of yet, however Underkofler and Hickey (1) mention that the commercial production of lactic acid has been carried out on a commercial unit. No references are given.

The liquid-liquid extraction of lactic acid has been proposed from time to time as a purification method (24, 38, 86, 115, . ., 121).

Jeneman's method (24) has been described previously.

Smith and Claborn (38) mention that no suitable solvent extraction method had been developed as of 1939.

Leonard, Peterson and Johnson (86) have done an extensive study of liquid-liquid extraction of lactic acid from fermented sulfite waste liquors. They have reported the distribution coefficients for a large number of systems.

Ratchford, et al. (117) studied the liquid-liquid extraction of lactic acid from a water solution by amine-solvent mixtures. Their experiments show that lactic acid can be extracted effectively from the water solution as the tertiaryamine salt. However, more work has to be done on the recovery of the acid from the solvent phase in order to develop a continuous process.

Weiser (121) performed a very detailed investigation of the distribution ratios of lactic acid between water and a considerable number of organic solvents. The effect upon the distribution ratio, K, of various inorganic salt additives and the complete ternary solubility envelope for lactic acid-water-isoamyl alcohol at 25°C and 49.5°C was

reported. In general, alcohols were found to be the best overall extracting agents for lactic acid. Isoamyl alcohol was found to be the less selective for the common sugar impurities. The steam distillation recovery of lactic acid from the solvent is discussed and a complete flow diagram for the proposed lactic acid purification process is given.

The literature search was very fruitful, especially with respect to the fermentation, extraction and distillation phases of the research program. Its usefulness will be discussed later.

OBJECTIVE

The objective of the research program was to determine, in a quantitative experimental manner, the feasibility of developing a basicly new continuous process for the manufacture of lactic acid.

A simplified flowsheet of the proposed process is given in Figure 2. It shows a fermentor, a solvent extraction unit, a solvent stripping column and a solvent recovery section. The process is based upon continuous fermentation, extraction and recycling of the fermented liquor.

Although not shown in Figure 2, it might be necessary to place a fermented liquor purification unit downstream from the fermentor. It might also be necessary to incorporate a unit to treat the raffinate prior to recycling it back to the fermentor, where:

$L_1 = L_2$ = Rate of removal of fermented liquor, liters per hour.

X_1 = Acid lactate concentration in the fermented liquor in grams per liter.

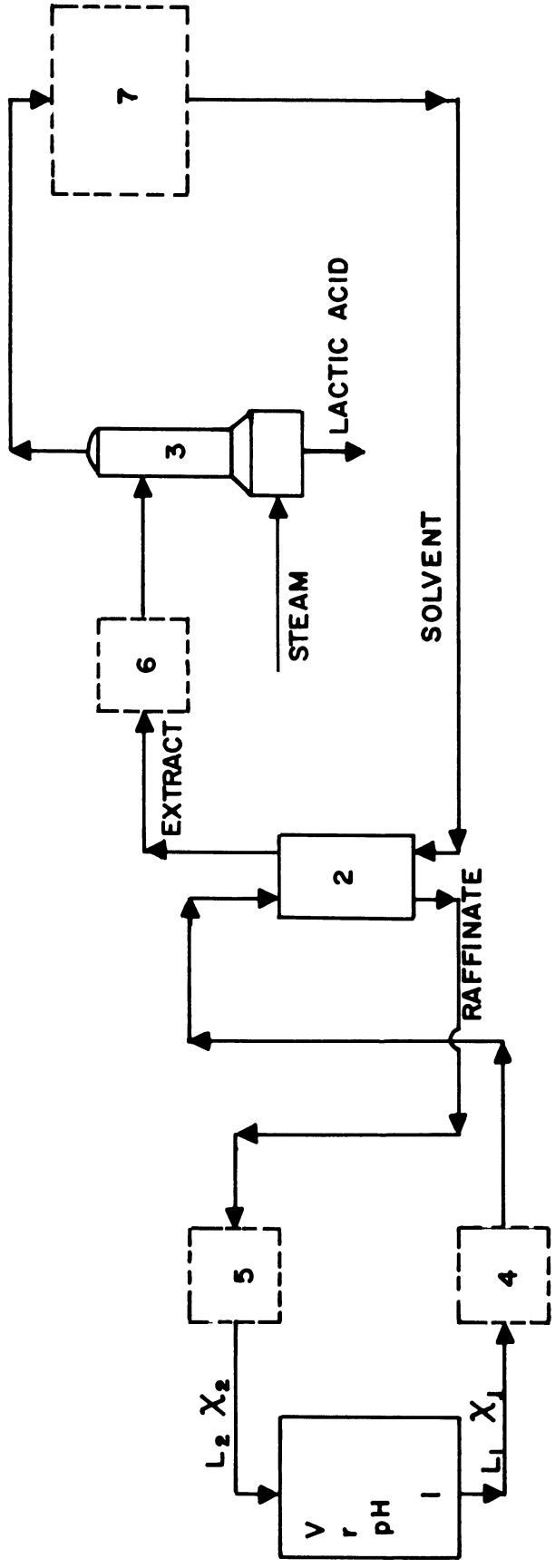
X_2 = Acid lactate concentration in the raffinate being recycled back to the fermentor, grams per liter.

r = Acid lactate fermentation rates at the pH at which the fermentation is being performed. Rate expressed as grams of acid lactate fermented per liter of fermenting liquor per hour of fermentation time.

V = Volume of liquor fermenting in the fermentor. Liters.

The acid lactate balance, for any constant pH at which the fermentor is operated shows that

$$L_1 X_1 - L_2 X_2 = r V$$



UNITS

1 – FERMENTOR
2 – EXTRACTOR
3 – SOLVENT STRIPPER
4 – FERMENTED LIQUOR PURIFICATION
5 – RAFFINATE PURIFICATION
6 – EXTRACT SETTLING
7 – SOLVENT RECOVERY

Figure 2. Schematic Diagram of Proposed New Process for the Manufacture of Lactic Acid

or

$$L_1 (X_1 - X_2) = r V$$

then

$$\frac{L_1}{V} = \frac{r}{X_1 - X_2}$$

which expresses the rate at which the fermented liquor must be removed from the fermentor in liters per hour per liter of fermenting liquor.

The process consists of four very distinct operations, namely: fermentation, extraction, recirculation and solvent stripping. Therefore, the research will be subdivided into each of these individual phases.

What follows is a discussion of the factors to be considered during the experimental program with respect to each individual operation of the process.

A. Fermentation

The process calls for the sugar solution to be fermented in such a way as to obtain the lactic acid in the acid form rather than as the salt which is the case in the present day process.

The pH of the fermenting liquor will need to be controlled within a range at which the metabolism of the microorganism will not be inhibited. The fermentation will contain a buffer which will not neutralize the acid being liberated. At the same time the buffer will restrict somewhat the rate of decrease of the pH value during the course of the fermentation This method of operation is considerably different to the present day fermentation process in which the pH is controlled at a constant value, usually ranging from pH 5.5 to pH 5.8.

Also, the present day methods make calcium lactate which needs to be purified and acidified in order to obtain the lactic acid while the proposed method makes lactic acid as such.

The buffer agent or agents must meet the following three specific requirements:

1. It cannot poison or inhibit the metabolism of the microorganism.

2. It must be able to control the pH of the fermentation within a practical range.

3. It must not neutralize the acid as it is formed.

The hydrogen ion concentration is produced by the dissociation of the lactic acid formed by the organism. It may be considered to be the result of the metabolism of the organism and it has been looked upon as one of the major factors limiting the growth of the fermentative bacteria.

The lactic acid produced during the course of a normal unbuffered fermentation will increase the values of both the hydrogen- and lactate-ion concentrations. The hydrogen-ion concentration does not change in cultures buffered to a constant pH. However, the undissociated lactic acid will accumulate in both cultures.

Let us consider the equilibrium existing between the ions and the undissociated molecules in a water solution of lactic acid.

$$HL \rightleftharpoons H^+ + L^- \qquad (1)$$

where H^+ and L^- represent the hydrogen and lactate-ion concentrations respectively. The concentration of the undissociated lactic acid is

represented by HL. The concentrations will be expressed in terms of gram-moles per liter of solution.

According to the mass action law we may define the dissociation constant as:

$$K = \frac{[H^+]\,[L^-]}{[HL]} \tag{2}$$

or

$$[HL] = \frac{[H^+]\,[L^-]}{K} \tag{3}$$

The $[L^-]$ term represents all of the lactate ion present. It is composed of the lactate ion, $[L_1^-]$, corresponding to the dissociated acid and whatever lactate ion, $[L_2^-]$, might be present from another source.

$$[L^-] = [L_1^-] + [L_2^-] \tag{4}$$

Therefore:

$$[HL] = \frac{[H^+]\,[L_1^- + L_2^-]}{K} \tag{5}$$

$$[HL] = \frac{[H^+][L_1^-]}{K} + \frac{[H^+][L_2^-]}{K} \tag{6}$$

In order to have an idea of the increase in concentration of the undissociated acid with respect to the hydrogen-ion concentration, let equation (6) be differentiated with respect to $[H^+]$ and since $[L_1^-] = [H^+]$,

$$\frac{d\,[HL]}{d\,[H^+]} = \frac{d\,[H^+]^2}{K\,d\,[H^+]} + \frac{d\,[H^+][L_2^-]}{K\,d\,[H^+]} \tag{7}$$

In the case of a fermentation performed at constant pH by the continuous neutralization of the liberated acid:

$$\frac{d\,[HL]}{d\,[H^+]} = 0 \tag{8}$$

since the hydrogen ion concentration would be a constant.

However, when the fermentation does not have any pH control:

$$\frac{d\,[HL]}{d\,[H^+]} = \frac{2\,[H^+]}{K} \qquad (9)$$

If we consider a fermentation which does not have any pH control but contains a certain concentration of lactate ion not derived from the dissociation of the lactic acid being liberated, from (7) we obtain:

$$\frac{d\,[HL]}{d\,[H^+]} = \frac{2\,[H^+]}{K} + \frac{[L_2^-]}{K} = \frac{2\,[H^+] + [L_2^-]}{K} \qquad (10)$$

Equation (10) shows that there is a possibility in increasing the concentration of the undissociated lactic acid by incorporating a certain amount of lactate-ion into the fermenting liquor. It also shows that it is not necessary to neutralize the acid being liberated by the organism. Since the hydrogen-ion concentrations will range from 10^{-3} to 10^{-6} gram-moles per liter it might be possible to eliminate its effect by making the $[L_2^-]$ concentration sufficiently high.

Let us say that the microorganism is able to withstand a pH of 3.16 without any buffer. Therefore, from the definition of

$$pH = \log \frac{1}{H^+} \qquad (11)$$

we can determine the molar concentration of the hydrogen ion as:

$$pH = 3.16 = \log \frac{1}{H^+}$$

$$\frac{1}{H^+} = 1.445 \times 10^3$$

$$[H^+] = 6.92 \times 10^{-4} \text{ gram moles per liter.}$$

Since

$$[H^+] = [L_1^-]$$

$$[HL] = \frac{(6.92)^2 \times 10^{-8}}{K}$$

$$= \frac{4.78 \times 10^{-7}}{K} \text{ gram moles per liter.}$$

A value for the dissociation constant $K = 1.38 \times 10^{-4}$ has been reported (140,145).

Therefore:

$$[HL] = 3.47 \times 10^{-3} \text{ gram moles per liter}$$

which is too low a concentration for any practical consideration. Equation (3) shows that the concentration of the undissociated acid can be increased either by depressing K, or by raising the $[H^+]$ and $[L^-]$ values. However, the value of K cannot be altered. On the other hand the value of $[H^+]$ cannot be raised further since it would cause a lower pH value than the organism would be able to withstand. Let us consider the $[L^-]$ value. By the definition of the dissociation constant, we can take into consideration all of the ionized lactate ion present in the solution Therefore we may increase it to such values that the portion due to the dissociated acid will be negligible and we would be using equation (5). $[L_2^-]$ represents the concentration of the lactate ion which will be added to the media in the form of readily ionizable lactic acid salts. Assuming that the organism will be able to carry the fermentation down to pH 3.16, when the media is 0.1 N $[L_2^-]$:

$$[HL] = \frac{[6.92 \times 10^{-4}][6.92 \times 10^{-4} + 0.1]}{1.38 \times 10^{-4}}$$

$$= 5.01 \times 10^{-1} \text{ gram moles per liter}$$

which is over 100 times larger than the lactic acid concentration without pH control.

The above analysis shows that it might be possible to make use of the Common Ion Effect that the lactic acid salts have upon the extent of dissociation of lactic acid.

Industrial experience has shown that calcium lactate does not affect the fermentation, otherwise calcium carbonate would not be used as a buffer agent. However, the experimental program will cover the utilization of a number of lactate salts, such as: sodium, potassium, lithium and magnesium.

Any study concerning the behavior of the fermentation should also include the study of a number of variables, namely: type of nutrients and concentrations, temperature levels, substrate concentration, presence of the solvent used for the extraction and the removal of the nutrients during the extraction.

While performing the studies on the fermentation it will be very desirable to determine whether there are different limits of the concentration of undissociated acid with different types of fermentative bacteria.

B. Extraction

The process calls for extraction of the acid lactate present in the fermented liquor.

A suitable solvent must be selected in order to make the extraction step practicable. There are several principles which can be used as a guide in the selection. Some of them will probably conflict and a compromise will need to be made.

The first solvent property to be studied will be the selectivity. This refers to the ability of a solvent to extract one component

of a solution in preference to another. The fermented liquor will have a high concentration of lactate salts in addition to the acid lactate. Thus the liquid-liquid system will consist of lactic acid, lactate salt, water, sugar, and solvent. This makes it a five component system. It will be of the utmost importance to find a solvent which will have a great selectivity for lactic acid since a slight ratio of lactic acid to lactate salt extracted will be enough to render the process impracticable. Therefore, it will be desirable to choose a solvent whose capacity for lactate salts and other impurities will be practically nil.

The second solvent property to be studied will be the recoverability from the streams resulting from the extraction step. The recovery of the solvent is very important since it will avoid the contamination of the products and at the same time it will permit reuse of the solvent after suitable purification. In almost every liquid-liquid extraction process, the solvent recovery is performed by fractional distillation. This points to the importance of choosing a solvent having a high relative volatility as compared to lactic acid. Also the latent heat of vaporization of the solvent should be as low as possible.

Lactic acid will be the less volatile component of the solvent phase. Therefore it will be desirable to use a solvent which will require the lowest solvent to liquor ratio since the amount of solvent to be vaporized will be considerably larger than the fermented liquor being extracted.

The difference in densities of the contacted phases should be as large as possible, otherwise the rates of disengaging the immiscible layers and the capacity of the extraction equipment will be decreased.

It is also important to make sure that there will be an appropriate density difference between the solvent- and raffinate-phases throughout the entire range of contemplated operation.

Other solvent properties which will help in the selection include: surface tension, chemical reactivity with lactic acid, and solubility in the fermented liquor. However, it might be desirable to consider the following properties with respect to the ease of storage of the solvent: corrosiveness, viscosity, vapor pressure, freezing point, inflammability, toxicity, cost, and availability.

C. Distillation

The process calls for the isolation of the lactic acid that has been extracted.

Because lactic acid has a low volatility, its isolation may be performed without much difficulty by fractional distillation of the solvent. The solvent will be obtained as the Distillate and the lactic acid as the Bottoms of the fractionating column.

There is every indication that the distillation step will be a relatively simple operation. However, it will be necessary to determine the conditions at which the distillation will be performed in order to obtain the best final product.

D. Recirculation

The process calls for recirculation of the extracted liquor.

The raffinate-phase stream leaving the extraction unit will contain most, if not all, of the lactate salts used as buffer agents during the fermentation. This stream will also contain any excess sugar which might be completely fermented if it is returned to the fermentor. There is also the possibility that the extracted liquor still contains

most of the nutrients which were required for the fermentation. Thus it might be possible to continue the fermentation in the extracted liquor by merely adding sugar at the rate at which it is being fermented and extracted. All of these point to the desirability of recirculating the extracted liquor; however it might be necessary to submit it to some sort of purification.

It is expected that the solvent will be soluble to some extent in the extracted liquor. The fermentative-bacteria might resent its presence thus making it necessary to reduce the solvent concentration level or to remove it completely.

It is not expected that the solvent will extract the nutrients from the fermented liquor. It might be necessary to clarify the liquor prior to the extraction step due to its content of the fermentation impurities.

Any purification performed in the liquor will remove or destroy a portion, if not all, of the organic nutrients. This being the case, it will be necessary to refortify the extracted liquor before recycling it back to the fermentor.

It might be possible to perform the extraction without any previous purification of the fermented liquor. However, in this case the solvent might cause the "coagulation" of the cellular mass of the organisms and of the organic nutrients. The phase on which the coagulum settles, whether the raffinate or solvent, will depend on its density. If the "coagulation" affects the nutritive value of the organic nutrient, it will be necessary to refortify the extracted liquor prior to its recycling.

Also, the accumulation of the "coagulum" might affect the phase equilibrium of the liquid-liquid extraction system. The coagulum might accumulate at the interface to such an extent that portions of it might be extracted in the solvent phase thus affecting the quality of the product.

It is expected that chances for contamination of the recycled media will be enhanced by the number of processing steps through which it is carried. Thus, it might be necessary to sterilize it prior to returning it to the fermentor.

METHODS AND MATERIALS

The objectives of the experimental program called for experimental work to be done in four distinct phases or steps of the proposed process. The experimental work performed on each phase will be discussed separately.

A. Fermentation

1. Physical equipment

a. Fermentors: The fermentors consisted of Florence Pyrex glass flasks, each one having a capacity of 2000 ml. They were stoppered with cotton plugs before they were steam sterilized.

b. Rocker mechanism: The fermentor flasks were subjected to the slight agitation of a rocker mechanism having 48 cycles per minute and a stroke of 6 inches. The length of the stroke could be varied in two-inch increasements by moving the connecting rod along the excentric. The box of the rocker measured 14-1/2 inches wide, 27 inches long and 7 inches deep. It was large enough to hold eight of the fermenting flasks at any one time. The box was supported on four ball-bearing rollers which ran on two one-inch structural steel channels. It was connected to an excentric which was directly attached to the drive shaft of a gear reduced 1/2 HP electric motor. The motor , box and box runners were mounted as a single unit. The unit was located inside the incubator in such a way so as to permit the continuous outside air cooling of the motor which might have otherwise burned out. The rocker would run continuously during the entire course of any one of the fermentations.

c. Incubator: The incubator used during the fermentations consisted of an insulated box measuring 6 feet high, 6 feet long and 3-1/2 feet deep. The insulation material consisted of one inch-thick Cello-Tex boards which were nailed to a wooden frame made of 2 x 2 wood members. The entrance of the box consisted of a full height door, 3 feet wide. It was large enough to walk into for the purpose of removing the rocker from the inside. The box also contained a small access door to an inside shelf which was used for the every day maintenance of stock cultures. The incubator box proved to be quite satisfactory. It was kept at constant temperature. The heating element consisted of a 640 watt (110 volt) electrical element which was controlled by means of a bi-metallic couple set at the desired temperature. In order to prevent the burning of the bi-metallic couple contact points, they were by-passed by a 10 amp (110 volt) solenoid which was actuated by a cut-out switch having a 60 second time-delay interval. A six-inch desk-fan was placed behind the heating element. This was operated continuously in order to recirculate the warm air around the box and to maintain a constant temperature throughout the entire box. A thermometer immersed in a water flask, simulating a fermentor, indicated a temperature of 39.5°C during the course of a fermentation when the incubator was set at a temperature of 40°C.

d. pH meter: The pH values were measured by means of a Beckman model H-2 glass electrode pH meter.

2. Nutrients

It has been shown that most of the carbohydrate media used for fermentations must be supplemented with inorganic salts (92) and nitrogeneous substances in order to supply the nitrogen required by

the organism. A number of investigators (72, 87, 104) have shown that malt sprouts furnish the organic growth factors required by the lactic acid fermenting bacteria. The malt sprouts are by-products of the malting process of barley grain. The grain is germinated under controlled temperature and humidity conditions which promote desired enzymatic reactions in the grain. After the grain has germinated it is air dried at a temperature of 55°C. The rootlets, which are called malt sprouts, are separated after the drying operation (150). They are extensively used in the fermentation industry and as supplements for cattle feed. The malt sprouts used in this research program were supplied by the Froedtert Grain and Malting Company, Inc. of Milwaukee, Wisconsin.

Malt sprouts were the only organic nutrients used during the course of this investigation. Two methods were used in adding the sprouts to the media. One of them consisted in the direct addition of the desired weighed amount to the mash. The other consisted of making an extract by boiling the sprouts in 500 ml of water for 5 minutes and then filtering through a clean cloth. The filtrate was used for making the media. This method proved quite useful during some of the extraction experiments.

MacLeod and Snell (92) have shown that in order to obtain the normal growth of lactic acid fermenting bacteria it is necessary to have certain inorganic ions present in the fermentation. These ions are either essential or stimulatory to the organism. Following Gillies (104) work, the ions have been incorporated in the following stock solutions which were renewed every two weeks.

Solution A: Stored at 5°C.

20 gms $MgSO_4 \cdot 7\ H_2O$
1 gm $FeSO_4 \cdot 7\ H_2O$
1 gm $MnSO_4 \cdot H_2O$
2 drops conc. HCl
Distilled water to 100 ml.

Solution B: Stored at 5°C.

25 gms sodium acetate
Distilled water to 200 ml.

Solution C: Stored at room temperature.

25 gms Na_2HPO_4
Distilled water to 200 ml.

Solution D: Stored at 5°C.

25 gms $(NH_4)_2HPO_4$
Distilled water to 200 ml.

3. Substrate

The substrates used during the fermentations included dextrose, sucrose and sugar cane black strap molasses. The molasses was supplied by the Eastern Sugar Associates and the Central Mercedita, Inc., both Puertorican concerns. The very first fermentations were performed with molasses, however it soon became apparent that it would be necessary to deal with the fundamentals of fermentation. Hence, most of the experimental work was performed with dextrose as the substrate.

4. Buffer agents

Salts of lactic acid were used as buffer agents. Different concentrations of sodium, lithium, potassium, magnesium, and calcium lactate were used. The sodium and the calcium lactate were bought at the Chemistry Store of the University of Michigan. They were of the best grades available commercially. However, lithium, potassium and

magnesium lactates were prepared from reagent grade alkalies and reagent grade lactic acid.

5. Fermentation mash

The fermentation mash consisted of the desired amounts of substrate, lactate salts and malt sprouts. In addition it contained 5 ml of salt solution "A" and 10 ml of each of salt solutions "B", "C" and "D". It was made up to a volume of 1500 ml with distilled water.

The main variables to be studied were the concentration of substrate, lactate salt and malt sprouts. The usual procedure was to hold two of them constant while varying the third one. Usually, a solution of 6000 ml was prepared containing the appropriate amounts of all the factors that it was desired to hold constant. After thorough mixing and adjustment to pH 5.8-6.2, it was placed in four 2000 ml Florence pyrex glass flasks, each containing aliquots of 1500 ml. The variable factor was added in different amounts to each flask. The flasks were then stoppered with cotton plugs and sterilized, four at a time, at 121°C for 15 minutes. After sterilization the flasks were permitted to cool overnight in the rocker unit inside the incubator.

6. Fermentative bacteria

The homofermentative organism, Lactobacillus delbrueckii NRRL B-445 obtained from the Northern Utilization Research Branch of the United States Department of Agriculture at Peoria, Illinois, was used throughout this experimental study.

The culture in every day use was carried on a liquid medium which consisted of 30 gms glucose, 2.5 ml salt Solution A, 5 ml each of salt solutions "B", "C" and "D", 30 gms of Difco yeast extract and enough distilled water to make a volume of 1000 ml. The media was

adjusted to pH 5.8 and transferred in 10 ml aliquots to screw-cap test tubes each containing dry calcium carbonate which acted as buffer. These tubes were sterilized at 121°C for 15 minutes and stored in the refrigerator.

The culture was transferred every day and kept in the incubator at 40°C.

The stock cultures were carried as stabs on solid media prepared by adding one percent agar to the above described liquid medium. These cultures were transferred at three month intervals. After each transfer, they were incubated at 40°C for 24-48 hours and then stored at 5°C in the refrigerator.

The starter cultures were grown on the liquid medium described above. These media were innoculated with a loopful of the everyday 24 hour culture and incubated for 10-15 hours at 40°C. The liquid content of each screw-cap test tube was used to innoculate each fermentation.

The inoculum volume of 10 ml kept to a practical minimum the amount of calcium lactate being added to each fermentation when studying the buffer effect of different lactate salts.

7. Sampling

Each fermentation was sampled immediately after innoculation and when it was stopped.

The following sampling procedure was used. An aliquot of about 75 ml was poured aseptically from the desired fermentation into a 250 ml Erlenmeyer flask. The flask and the contents were cooled to 20°C and the 75 ml were filtered through a fine copper screen in order to remove the malt sprout solids.

An aliquot of 25 ml of the filtrate was pipetted into a 250 ml glass stoppered volumetric flask containing 25 ml of 1.0 N H_2SO_4. The sample and the acid were completely mixed by gentle swirling. According to Friedeman (149) this acid concentration will instantly stop all metabolic activity of the organism. The flask was set aside for about 15 minutes before diluting with distilled water. After dilution, the flask was thoroughly mixed and stored at 5°C in the refrigerator. This sample was used for lactate and sugar analyses.

Another 25 ml aliquot was pipetted into a clean dry 100 ml glass beaker. This sample was used for pH measurement and determination of the titrable acidity. These two measurements were performed at least every 24 hours starting from the time of inoculation on nearly every fermentation performed. In this way it was possible to follow the progress of the fermentations.

8. Lactate ion analysis

The total lactate ion concentration was determined by the Friedmann and Graeser (142) method. This involves controlled oxidation of lactic acid into acetaldehyde without further oxidation into acetic acid. The acetaldehyde is removed by distillation and bound as the bisulfite-acetaldehyde complex. Excess bisulfite is titrated to the starch end point with a strong iodine solution. The bound bisulfite is liberated with sodium bicarbonate, and sodium carbonate after which it is titrated with 0.01 normal iodine solution. Each milliliter of the 0.01 N I_2 solution required for the oxidation of bisulfite, is equivalent to 0.45 mg of lactic acid in the oxidized sample.

Friedmann and Graeser (142) were able to account for 99 percent of the lactate ion present in standard samples of known compositions. However, a number of investigators (104) have reported

recoveries ranging from 85 to 90 percent when analyzing standard samples of known concentrations. They utilized a correction factor corresponding to their recoveries in calibration runs in order to account for all of the lactic acid present.

The Friedmann and Graeser method is specific for lactic acid and can be used for its quantitative determination. However, there are certain precautions which must be taken in order to obtain the best results. For example, reducing sugars and proteinaceous matter interfere with the final results and must be removed from the sample to be analyzed. Their removal can be accomplished by copper hydroxide precipitation. According to Freeman and Morrison (148) it is not necessary to use more elaborate means when the fermented liquor contains 5 percent sugar or less. Friedmann and Graeser (142) also recommend the copper hydroxide precipitation for clarification purposes.

Friedmann and Graeser (142) recommend the use of colloidal manganese dioxide to oxidize and decarboxylate the lactic acid into acetaldehyde. Gillies (104) reported a number of difficulties which we have also encountered during its preparation. Potassium permanganate has been recommended (147) as the oxidizing agent. However, no information has been published referring to the percent recovery obtained with it. Recoveries ranging from 95 to 98 percent, if not higher, were obtained during the course of this research. The analytical procedure and equipment used was as follows.

The Kjeldhal oxidation - distillation apparatus was slightly modified as compared to the one that has been described (142). The stem of the dropping funnel used for the controlled addition of potassium permanganate was made of glass tubing having an outside diameter of 6 mm and an inside diameter of 1.0 mm. It extended to within

one-quarter of an inch from the liquid level in the 300 ml Kjeldahl flask. This modification avoided further oxidation of the acetaldehyde vapors into acetic acid, since there was no oxidizing agent dripping down the walls of the Kjeldahl flask. Small electric heaters rated at 110 volts and 250 watts were used instead of Bunsen burners. The heaters permitted close control of the rate at which the samples were heated.

The acidified sample from the refrigerator was warmed to 20°C before use. Twenty-five ml were then pipetted into a 250 ml glass stoppered volumetric flask. Ten ml of 20 percent copper sulfate solution were next added and the neck of the volumetric flask was washed with 20 ml of distilled water. The contents were mixed by gentle swirling and ten ml of 20 percent calcium hydroxide suspension were added. The neck of the flask was again washed with 20 ml of distilled water and the contents were completely mixed by gentle swirling being careful not to develop a foam. The flask was made up carefully to the mark and the contents were thoroughly mixed. It was then allowed to stand for 1-1/2 hours during which time the blue copper hydroxide precipitate settled. The supernatant liquor, which was water clear, was used for the lactate analysis.

An aliquot containing not more than 5 mg of the lactate ion was pipetted into the 300 ml Kjeldahl flask, which contained 10 ml of the phosphoric acid solution and a pinch of talcum. The neck of the flask was washed down with enough distilled water to make the total volume approximately 100 ml. The flask was connected to the apparatus and the electric heater was placed under it. The heating rate was such that the flask contents started boiling in 3-1/2 to 4 minutes.

It was allowed to boil for 2 minutes before addition of potassium permanganate was started. Approximately 0.01-0.02 normal potassium permanganate was used. It was added in a dropwise fashion so that there was never an excess during the first 10 minutes of the oxidation-distillation procedure which was timed to take 15 minutes. There was always an excess of permanganate during the last five minutes. This excess was added at such a rate that the liquid never stopped boiling.

The Erlenmeyer flask which was receiving the distillate and which contained the excess bisulfite was removed after the 15 minute oxidation-distillation period was over. The tip of the condenser, which was immersed in the bisulfite, was thoroughly rinsed with a thin stream of distilled water, returning the rinsings to the flask. Then the flask and contents were cooled to 20°C and the analytical procedure was continued as described by Friedmann and Graeser (142).

The idea of extending the stem of the dropping funnel and of using electric heaters was arrived at after a considerable number of trial runs with standard lactate samples of known compositions. The trial runs also showed that approximately 80 percent of the lactate present was oxidized and distilled during the first 10 minutes. This indicated that the presence of an excess of permanganate during this time could account for the conversion of a portion of lactate into acetic acid.

The following trial runs were performed on a standard lithium lactate solution containing 0.09371 mg of lactate ion/ml. They demonstrate the marked improvement resulting from the equipment modification and the use of potassium permanganate instead of the manganese dioxide.

Results when using an excess of 0.01 N MnO_2 from beginning to end of oxidation-distillation along with electric heaters and short-stem dropping funnels which permitted the MnO_2 to drip down the wall of the flasks.

Trial I

Volume of Sample : 25 ml = 2.343 mg of lactate ion

Volume of 0.01 N Iodine for sample = 4.415 ml

Volume of 0.01 N Iodine for water blank = 0.150 ml

Net 4.265 ml

Conversion factor = 0.45 mg lactate/ml 0.01 N Iodine

mg lactate ion = (4.265) (0.45) = 1.919

Percent recovery = $\frac{1.919}{2.343}$ (100) = 81.8 percent

Trial II

Volume of Sample: 25 ml = 2.343 mg of lactate ion

Volume of 0.01 N Iodine for sample = 4.730 ml

Volume of 0.01 N Iodine for water blank = 0.150 ml

Net 4.580 ml

mg lactate ion = (4.58) (0.45) = 2.061

Percent recovery = $\frac{2.061}{2.343}$ (100) = 87.9 percent

Results when using an excess of 0.01 N $KMnO_4$ from beginning to end of oxidation-distillation, electric heaters and short-stem dropping funnel which permitted the permanganate to drip down the walls of the flask.

Trial I

Volume of Sample: 25 ml = 2.343 mg lactate ion

Volume of 0.01 N Iodine for sample = 4.86 ml

Volume of 0.01 N Iodine for water blank = 0.15 ml

Net 4.71

mg lactate ion (4.71) (0.45) = 2.12 mg

Percent recovery = $\frac{2.12}{2.343}$ (100) = 90.5 percent

Trial II

Volume of Sample: 25 ml = 2.343 mg lactate ion

Volume of 0.01 N Iodine for sample = 4.61 ml

Volume of 0.01 N Iodine for water blank = 0.15 ml

Net 4.46 ml

mg lactate ion = (4.46) (0.45) = 2.00 mg

Percent recovery = $\frac{2.00}{2.343}$ (100 = 85.3 percent

Results when using 0.01 N $KMnO_4$, electric heaters and long-stem dropping funnels. An excess of $KMnO_4$ during last 5 minutes of oxidation-distillation.

Trial I

Volume of Sample: 25 ml = 2.343 mg of lactate ion

Volume of 0.01 N Iodine for sample = 5.19 ml

Volume of 0.01 N Iodine for water blank = 0.15 ml

Net 5.04 ml

mg lactate ion = (5.04) (0.45) = 2.27 mg

Percent recovery = $\frac{2.27}{2.343}$ (100) = 96.9 percent

Trial II

Volume of Sample: 25 ml = 2.343 mg of lactate ion

Volume of 0.01 N Iodine for sample = 5.200 ml

Volume of 0.01 N Iodine for water blank = 0.150 ml

Net 5.050 ml

mg lactate ion = (5.06) (0.45) = 2.27 mg

Percent recovery = $\frac{2.27}{2.343}$ (100) = 96.9 percent

Trial III

Volume of Sample: 25 ml = 2.343 mg of lactate ion

Volume of 0.01 N Iodine for sample = 5.16 ml

Volume of 0.01 N Iodine for water blank = 0.15 ml

Net 5.01 ml

mg lactate ion = (5.01) (0.45) = 2.25 mg.

Percent recovery = $\frac{2.25}{2.343}$ (100) = 96 percent

The concentration of the lactic acid formed by fermentation was determined with a material balance. The initial lactate concentration at the time of inoculation was subtracted from the final lactate concentration present when the fermentation was stopped.

Lactate Determination

Fermentation No. 36-F-6

Lithium lactate added as buffer: approximately 10 gms.

Initial conditions:

Samples analyzed: 5 ml of the supernatant liquid from the copper hydroxide treatment. At this point the original sample had been diluted 100 times.

Ml of 0.01 N Iodine solution required:

I		II	
Final	2.48	Final	3.00
Original	1.90	Original	2.48
Net	0.58	Net	0.52

Average ml of 0.01 N Iodine solution required = 0.55

Ml of 0.01 N Iodine solution for water blank = 0.10

0.45

$$\text{Lactate ion concentration} = \frac{(0.45)(0.45)(100)}{(.96)\ (5)}$$

$$= 4.22 \text{ gms/liter}$$

Final conditions:

Samples analyzed: 5 ml of the supernatant liquid from the copper hydroxide treatment. At this point the original sample had been diluted 100 times.

Ml of 0.01 N Iodine solution required:

I		II	
Final	2.60	Final	4.37
Original	0.74	Original	2.60
Net	1.86	Net	1.77

Average ml of 0.01 N Iodine solution required = 1.82

Ml of 0.01 N Iodine solution for water blank = 0.10

1.72

$$\text{Final lactate ion concentration} = \frac{(1.72)(0.45)(100)}{(0.96)\ (5)}$$

$$= 16.1 \text{ gms/liter}$$

Fermented acid lactate = 16.1 - 4.22

= 11.88 gms/liter

9. Sugar analyses

The concentration of sugar was determined by the method of Shaffer and Somogyi (143). It consisted of the usual copper reduction method involving iodometric titration of the reduced copper followed by titration of the excess iodine present in the copper reagent with sodium thiosulfate. The copper reagent used has been described by Somogyi (146) in 1952.

The method is not specific for fermentable sugars. The sugar values obtained represent the copper reducing capacity of the analyzed sample as compared with that of glucose. This capacity is expressed in terms of glucose concentrations by using a conversion factor obtained from calibration analyses of the copper reagent using reagent grade glucose.

The starch indicator was prepared as follows. Two grams of potato starch were suspended in 10 ml of water and added to 450 ml of boiling distilled water. The solution was boiled for 10 minutes. Five drops of toluene and 2 grams of potassium iodide were added upon cooling. This starch solution proved to be quite stable when kept in the refrigerator. One ml of the solution was regularly used for each determination.

A sample calculation will demonstrate how the sugar concentrations were determined in fermentation No. 36-F-6.

Sugar Determination

Fermentation No. 36-F-6

December 16, 1955

The sodium thiosulfate solution used for titration of the excess iodine from the copper reagent was standardized against a 0.10 N potassium iodate solution. An aliquot was diluted to 500 ml in order to obtain a 0.005 N thiosulfate solution. The aliquot was measured with the 25 ml standard burette used for the sugar analyses.

The sample was treated as follows. The acidified sample kept in the refrigerator was warmed to 20°C. Twenty-five ml were pipetted into a 100 ml glass stoppered volumetric flask containing 10 ml of a 10 percent lead acetate solution and completely mixed by gentle swirling. The flask was made up to volume and thoroughly mixed. A portion of the liquid was centrifuged and 25 ml of the clear supernatant were pipetted into a 100 ml glass stoppered volumetric flask containing 10 ml of a 10 percent neutral potassium oxalate solution. The flask was made to volume and the contents were then completely mixed. During a standing period of 1-1/2 hours a white lead oxalate precipitate settled out. Five ml of the water clear supernatant were used for the sugar analysis. At this point the original sample had been diluted 160 times. The blank contained 5 ml of distilled water.

Sugar concentration at time of inoculation:

Blank		Unknown Sample		Difference
15.00	15.00	9.75	19.60	15.00
-0.00	-0.00	-0.00	-9.85	-9.75
15.00	15.00	9.75	9.75	5.25 ml
Average:	15.00 ml	Average:	9.75 ml	

The difference in the 0.005 N sodium thiosulfate titration was 5.25 ml. According to the copper reagent calibration curve it represented 0.84 mg of reducing substance, expressed as glucose, in the 5 ml analyzed. This was converted into grams per liter of fermentation mash as follows:

$$\frac{0.84}{5}(160) = 26.88 \text{ grams/liter}$$

Sugar concentration at end of fermentation:

Blank		Unknown Sample		Difference
15.00	15.00	12.20	24.60	15.00
-0.00	-0.00	-0.00	-12.25	-12.27
15.00	15.00	12.20	12.35	2.73 ml
Average:	15.00 ml	Average:	12.27 ml	

The difference in the 0.005 N sodium thiosulfate titration was 2.73 ml. According to the copper reagent calibration curve it corresponded to 0.44 mg of reducing substance, expressed as glucose, in the 5 ml analyzed. This was converted into grams per liter as follows:

$$\frac{0.44}{5}(160) = 14.08 \text{ grams/liter}$$

Initial concentration = 26.88 gms/liter

Final concentration = 14.08 gms/liter

Total sugar utilized by organisms = 12.80 gms/liter

Total acid lactate fermented = 11.88 gms/liter

Yield $= \frac{11.88}{12.80}(100)$ = 92.8 percent

It should be stated at this point that the sugar analyses were not performed on all of the fermentations reported herein. From the point of view of the development of the process it was not of the utmost importance to perform such analyses. The importance of the fermentation phase of the research was to establish how much acid lactate the organism was able to produce and withstand as long as it had more than the necessary amount of substrate.

Kempe (102) and Gillies (104) definitely proved that the homogermentativeness of Lactobacillus delbrueckii varies slightly with the pH at which the fermentation is performed. However, the variation is not so large that it will affect the total amount of acid lactate fermented and withstood by the organism as long as there was enough sugar present to produce that total amount of acid lactate.

B. Solvent Extraction of the Acid Lactate

1. Physical equipment

a. Separatory funnels: All of the extraction experiments were performed in Pyrex glass separatory funnels which had a capacity of 500 ml. The stop cocks and the glass stoppers were held tightly in place by means of stiff rubber bands.

b. Constant temperature water bath: All of the quantitative extraction experiments were performed at a temperature of 40°C. This temperature was obtained by means of a constant temperature water bath. The bath consisted of a circular pyrex glass container

measuring 30 cm in diameter and 30 cm in height. When in operation it contained 15 liters of water which were stirred by means of a three inch, propeller type, air driven stirrer. The water was maintained at a temperature of 40°C ± 0.10°C. The electrical heating element was turned off and on by means of a bi-metallic couple set at the desired temperature. The contact points were by-passed by a solenoid actuated by a cut-out switch having a one second delay interval. Temperature measurements throughout the bath did not show any appreciable differences.

c. Pan balance: All of the samples used in the quantitative extraction experiments were weighed on a tared pan balance which was accurate within two grams when weighing samples as large as 500 grams.

d. Timer: An interval timer was used for timing the shaking of the samples and the time interval between each shaking. The timer was also used during the oxidation-distillation procedure of the lactate analysis by the Friedmann and Graeser (142) oxidation method.

2. Theory

The literature survey revealed the very thorough experimental work done by Weiser (121). He published equilibrium data for the ternary system, lactic acid-water-isoamyl alcohol. While working with this system he was able to prove conclusively certain facts which are directly related to our objectives and experimental requirements. He reported no work with actual fermentation liquors. So a short discussion relating his results to our objectives is advisable at this point.

It was proven that the capacity of iso-amyl alcohol to extract sucrose and lactose is very low if not negligible. However, no

matter how low the concentration of the sugar extracted by the solvent may be, it will still appear in the final product as a brown color due to the caramelization of the sugar during the distillation step. He determined and published the distribution coefficients for sucrose and lactose. As previously discussed, the low selectivity of the solvent toward the sugars is one of our most important solvent requirements.

It was shown that the selectivity of the alcohol toward the mineral salts found in the fermentation liquor is negligible. It was also proven that the presence of the salts have a slight beneficial effect upon the distribution coefficient of the acid lactate between the extract and raffinate phases. These facts indicate that there might not be any difficulties encountered during the extraction due to the mineral salts used as growth requirements, namely the salts incorporated in solutions "A", "C" and "D".

It was definitely shown that the selectivity of iso-amyl alcohol is very high toward the acid lactate found in a water solution. However, no work was done with solutions containing a mixture of lactate salts and acid lactate.

Lactic acid of the plastic grade was obtained when the extract phases were steam distilled which substantiates original contention that it is possible to obtain the acid by distillation of the solvent. He obtained a very light brown product indicating that there was caramelization of the sugar extracted by the alcohol. No trouble was reported in removing the color with activated charcoal.

Weiser's (121) work indicates that iso-amyl alcohol might be a very suitable solvent for our purpose; that is, to extract acid lactate from a fermentation liquor which contains glucose and lactate

salts. All that remained to be done was to prove whether his equilibrium data was applicable to the case at hand.

The experimental work performed to prove the applicability of the data is based on the following theoretical aspects of liquid-liquid extraction.

Let Figure 3 represent the general right-triangular diagram of a ternary system. The curve RLEV represents the typical binodal-solubility curve enclosing an area of two liquid phases.

Let us consider streams (or quantities of material) S and F having the compositions shown in Figure 3. If these two streams are combined into a single stream the overall material balance would show that:

$$J = F + S \tag{1}$$

Similarly, for any single component in any one of the streams.

$$J\,x_J = F\,x_F + S\,x_S \tag{2}$$

Substituting (1) into (2) and simplifying

$$J = \frac{F\,x_F + S\,x_S}{F + S} \tag{3}$$

For component A

$$(x_A)_J = \frac{F(x_A)_F + S(x_A)_S}{F + S} \tag{4}$$

For component B

$$(x_B)_J = \frac{F(x_B)_F + S(x_B)_S}{F + S} \tag{5}$$

For component C

$$(x_C)_J = \frac{F(x_C)_F + S(x_C)_S}{F + S} \tag{6}$$

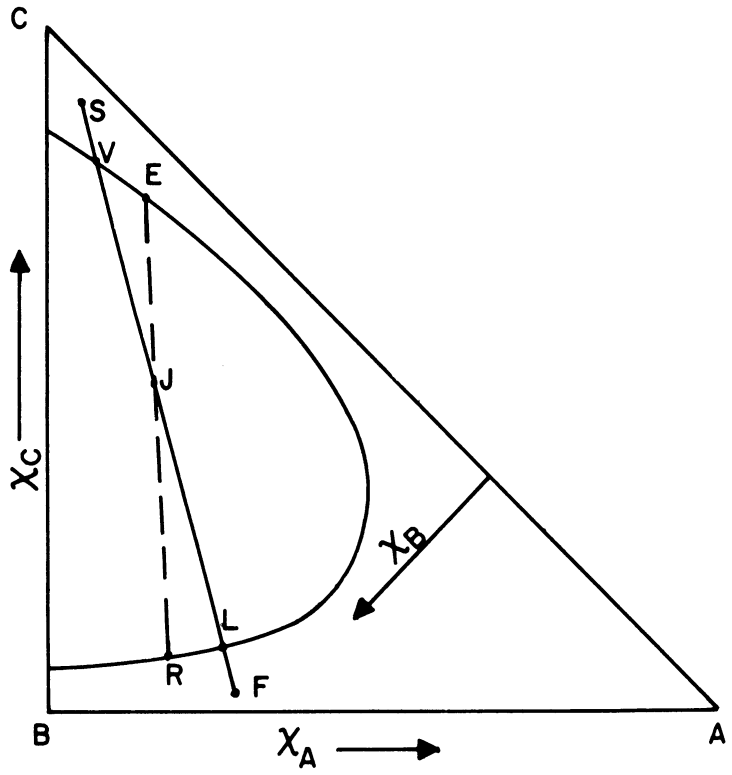


Figure 3. Typical Ternary Diagram for a Type I System

If the slope of the straight line connecting points x_F and x_J is the same as the slope of the straight line connecting x_F and x_S, it follows that the three points x_F, x_J and x_S are on the same straight line. Thus, a material balance such as equation (3) can be made by simply drawing a straight line through the points representing the compositions of any two of the three streams.

The slopes of the lines joining points x_F and x_J and connecting points x_F and x_S are

$$\frac{(x_C)_J - (x_C)_F}{(x_A)_F - (x_A)_J} \quad \text{and} \quad \frac{(x_C)_S - (x_C)_F}{(x_A)_F - (x_A)_S} \tag{7}$$

respectively.

Substituting the values of $(x_C)_S$ and $(x_A)_S$ from equations (6) and (4) into equation (7) and simplifying,

$$\frac{(x_C)_S - (x_C)_F}{(x_A)_F - (x_A)_S} = \frac{(F + S)(x_C)_J - (F + S)(x_C)_F}{(F + S)(x_A)_F - (F + S)(x_A)_J}$$

$$= \frac{(x_C)_J - (x_C)_F}{(x_A)_F - (x_A)_J}$$

The slope of the line between points x_F and x_J is the same as between points x_F and x_S. Therefore all three points must lie on the same straight line. The location of point x_J, relative to points x_F and x_S, is always between x_F and x_S when streams S and F are added to form stream J.

The linear distance between these three points is always dependent upon the quantities F and S. This may be demonstrated by simplifying and regrouping equation (3).

$$F x_J + S x_J = F x_F + S x_S$$

$$\frac{F}{S} = \frac{x_S - x_J}{x_J - x_F}$$

or

$$\frac{F}{S} = \frac{\overline{S\ J}}{\overline{J\ F}}$$

where $\overline{S\ J}$ and $\overline{J\ F}$ represent the linear distances from point S to J and from point J to F respectively.

Furthermore, equilibrium is established in an ideal stage, so that the two phase mixture J must separate into the extract and raffinate solutions E and R. They are the products of the extraction and are located on the binodal-solubility curve at opposite ends of the equilibrium tie line which passes through J. Their relative weights may be computed graphically in a similar manner to the $\frac{F}{S}$ ratio.

$$\frac{R}{E} = \frac{\overline{E\ J}}{\overline{J\ R}}$$

These theoretical aspects of liquid-liquid extraction furnish us with a method of determining the applicability of Weiser's data when extracting our fermented liquors.

First of all let us assume that his data is applicable to our case and proceed to prove the assumption in the following fashion.

A known weight of fermented liquor of a known acid lactate and solvent composition is thoroughly mixed with a known weight of pure solvent. The resulting two-phase mixture, denoted by J, will lie in the two-phase region enclosed by the binodal-solubility curve. After it has been mixed thoroughly, it is allowed to stand for an

appropriate length of time so that it will separate into the extract E and raffinate R phases when complete equilibrium is reached.

The acid lactate composition of the extract and raffinate phases will locate points E and R which supposedly will fall on the assumed binodal-solubility curve. The data will be applicable to our case only and only if the two following conditions are met.

a. The tie line joining point E and R must pass through point J.

b. The experimental value of the ratio $\frac{\text{Extract}}{\text{Raffinate}}$, found by weighing the separated phases, must be equal to the graphical ratio $\frac{J'R}{J'E}$ found by measuring the distances $\overline{J'R}$ and $\overline{J'E}$ along the tie line. The point J' represents the intersection point of the tie line with the S F line.

If these two conditions are met then it can be said that Weiser's data is applicable for the extraction for the fermented liquor. It will also indicate that the other components of the fermented liquor, namely glucose, buffer lactate, mineral salts and metabolic impurities do not significantly affect the equilibrium of the system, lactic acid - water - iso-amyl alcohol.

3. Experimental procedure

The experimental procedure for the quantitative extraction experiments was as follows:

a. General: The freshly fermented liquors were obtained from fermentations which had reached the maximum titrable acidity. For the extraction experiments they were filtered through a clean dry

piece of white cloth in order to remove the malt sprout solids. This filtration did not remove the bacteria.

The samples were weighed in 500 ml glass separatory funnels which were supported by a small test stand on the pan balance. Prior to weighing the samples, the balance was tared to the weight of the separatory funnels and the test stand. Samples of 100 grams of the fermented liquor were used throughout most of the quantitative extraction experiments. The iso-amyl alcohol was then weighed into the funnels. Definite ratios of solvent to fermented liquor were used. Usually three extractions were performed on each fermented liquor. The ratios of solvent to liquor used were 0.333, 1.00, and 2.00. Three tie lines were obtained in this way.

After the alcohol had been added to the sample, the stopcocks and glass stoppers of the funnels were held tightly in place by means of stiff rubber bands. The funnels were shaken for one minute and placed in the constant temperature bath which was set at 40°C. After the funnels had been in the water for one hour, they were thoroughly shaken four times for one minute with five minutes settling allowed between each shaking. They were then left in the water bath for four hours which was found to be the minimum time required for the two phases to reach equilibrium.

The separatory funnels were removed one at a time from the water bath and supported on the test stand on the pan balance. The balance was again tared and the raffinate phase was carefully drawn off. The weight of each phase was determined by difference from the original total weight. Each phase was analyzed for the components. Special attention was paid to the buffer lactate - acid lactate ratios.

b. Sampling and analytical procedures: The sampling and analytical procedures during the quantitative extraction experiments were as follows.

1. Original conditions before extraction: A 25 ml sample of the fermented liquor filtrate at 20°C was pipetted into a 250 ml glass stoppered volumetric flask which contained 25 ml of 1.0 N H_2SO_4. This sample was used to determine the sugar and total lactate present prior to the extraction. An equivalent sample had been taken at the time the fermentation was started. Thus the difference of the total lactates in the two samples was equivalent to the fermented acid lactate. Another 25 ml sample of the fermented liquor filtrate at 20°C was pipetted into a 100 ml glass beaker containing 25 ml of distilled water. This sample was titrated with a standard sodium hydroxide solution. The titration end point was determined with the Beckman Glass Electrode pH meter. Titration curves representing pH units vs. cumulative volume of the standard sodium hydroxide solution indicated that the titration end point fell in the pH range of 7.0 to pH 7.10. These titration values of the acid lactate were compared with the results obtained by the oxidation method. The comparison indicated that the acid lactate can be determined by titration to a pH value of pH 7.0 to pH 7.10. Gillies (104) found that the fermented lactate was equivalent to 88.1 percent of the titration values obtained during his fermentations. He titrated to pH 5.8. Kempe (103) later used this same value. Our work indicates that the fermented acid lactate as determined by the Friedmann and Graeser (142) method accounts for more than 98 percent of the titrable acidity.

2. Sampling and analysis of the raffinate phase: The raffinate phase was carefully drawn off the separatory funnel. A 25 ml sample was carefully pipetted into a 250 ml round bottom distillation flask. One hundred ml of distilled water were added and the contents were distilled until 50 ml had been collected as distillate. The distillate contained the iso-amyl alcohol originally present in the 25 ml of the raffinate phase. The residue (which contained the acid lactate, buffer lactate, sugar and matebolic impurities) was transferred quantitatively into a 150 ml beaker. All of the transferred material was titrated with a standard sodium hydroxide solution using a glass electrode pH meter to indicate the end point. The titration value accounted for all of the acid lactate in the 25 ml of the raffinate phase.

Another 25 ml of the raffinate phase were pipetted into a 100 ml glass beaker which contained 25 ml of distilled water. This sample was also titrated with the standard sodium hydroxide solution using the glass electrode pH meter to indicate the titration end point.

The titrable acidity, or acid lactate, obtained from the distilled sample agreed to within 1-1/2 percent of the values obtained by diluting the samples with 25 ml of distilled water.

Another 25 ml sample of the raffinate phase was pipetted into a 250 ml glass stoppered volumetric flask which contained 25 ml of 1.0 N H_2SO_4. The contents were mixed by gentle swirling. After thorough mixing, the flask was made up to volume with distilled water and placed in the refrigerator. This sample was used for determination of the sugar and total lactate present.

The buffer lactate was then determined by subtracting the acid lactate found by titration from the total lactate determined by the oxidation method.

The specific gravity of the raffinate phase was determined on all of the quantitative extraction experiments.

The acid lactate mass fraction of the raffinate phase was determined as follows:

$$\frac{\text{Titrable acidity as grams of acid lactate per liter}}{1000 \times \text{specific gravity}}$$

3. Sampling and analysis of extract phase: The separatory funnel was returned to the constant temperature bath as soon as the raffinate phase had been drawn off and weighed. Since the solubility of water in iso-amyl alcohol varies slightly with temperature, it was found that the extract phase would become cloudy upon cooling to room temperature. Some water would come out of solution and settle at the bottom of the separatory funnel. In addition a coagulum often formed on the extract phase. It was found that it was possible to agglomerate this coagulum further by swirling the extract phase. Following this, most of the coagulum settled to the bottom of the extract phase.

A 25 ml sample was carefully drawn from the clear extract phase. Care was taken not to draw any of the coagulum. The sample was carefully pipetted into a 250 ml round bottom distillation flask and 100 ml of distilled water were added. The contents were distilled until 75 ml had been collected. This was enough to remove all of the alcohol contained in the 25 ml of the extract phase. The 50 ml remaining in the flask were quantitatively transferred to a 150 ml glass beaker. All of the transferred material was titrated with the

standard sodium hydroxide solution. The titration end point was determined in the same fashion as when titrating the raffinate phase samples.

Another 25 ml sample was carefully drawn and pipetted into a 100 ml glass beaker containing 50 ml of distilled water. This was also titrated as the raffinate phase sample.

The titrable acidity, or acid lactate, obtained from the distilled sample agreed to within 0.5 percent of the values obtained by diluting the samples with 50 ml of distilled water.

Another 25 ml sample of the extract phase was carefully drawn and pipetted into a 250 ml round bottom distillation flask containing 100 ml of water. Seventy-five ml were distilled and the remainder was quantitatively transferred to a 250 ml glass stoppered volumetric flask containing 25 ml of 1.0 N H_2SO_4. The flask was made up to volume and completely mixed. It was then placed in the refrigerator. This sample was used for the total lactate and sugar determinations. It was necessary to remove the alcohol since it would have been impossible to dissolve it in 225 ml of water.

The specific gravity of the solvent phase was determined for each extraction experiment.

By the methods previously discussed it was possible to determine how much sugar and buffer lactate was being extracted along with the acid lactate by the iso-amyl alcohol.

The acid lactate mass fraction in the extract phase was determined as follows:

$$\frac{\text{Titrable acidity expressed as grams of acid lactate/liter}}{1000 \times \text{specific gravity}}$$

The oxidation method does not distinguish between the buffer and acid lactates. Thus, the buffer lactate was determined by subtracting the titrable acidity, expressed as grams of acid lactate per liter, from the total lactate concentration obtained by the oxidation method.

C. Distillation of the Extract Phase

The literature survey revealed the very thorough experimental work done by Weiser (121). As previously discussed, while he was working with the system, lactic acid - water - iso-amyl alcohol, he proved certain facts which are directly related to our objectives.

It was conclusively proven that lactic acid of the plastic grade can be obtained when the extract phase is steam distilled which substantiates our original contention that it is possible to obtain the acid by distillation of the solvent. The product obtained was of a very light brown color which was easily clarified with activated charcoal.

D. Recirculation

1. General

The experimental work involving recycling of the raffinate phase back to the fermentors involved the treating of the raffinate in different ways so as to determine the conditions under which the recirculation would be a feasible operation. Three general types of these experiments were performed and what follows is a short discussion of each.

Type 1: Vigorously growing, forty-eight hour fermentations were extracted with normal butyl and iso-amyl alcohols. The raffinates were reincubated without further treatment. They were checked at the

end of forty-eight hours to determine whether the fermentative bacteria had continued fermenting the remaining sugar.

Type 2: Vigorously growing, forty-eight hour fermentations were extracted aseptically with normal butyl and iso-amyl alcohol. The raffinate phases were each divided into three portions, one portion of each raffinate treated as follows and incubated:

a. This portion was stripped of alcohol, made back to original volume, sterilized, reinoculated and reincubated.

b. This portion was reinoculated and reincubated as such.

c. This portion was reincubated without any further treatment.

Each portion was left in the incubator for forty-eight hours after which time it was checked to determine whether the bacteria had continued fermenting the remaining sugar.

Type 3: A fermentation mash was prepared in the usual manner using an extract of fifteen grams of malt sprouts as nutrients. It was then extracted with iso-amyl alcohol; the alcohol was distilled off of the raffinate phase after which the raffinate was refortified with malt sprouts and sterilized. It was finally inoculated and placed in the incubator.

Regular fermentation mashes were inoculated and incubated. Different amounts of normal butyl and iso-amyl alcohol were added at different times during the fermentation in order to determine what inhibiting effect that the alcohols would have on the fermentative bacteria during the extraction step.

EXPERIMENTAL RESULTS

A. Fermentation

1. Effect of using different lactate salts as buffer agents

Four series of fermentations were performed in order to determine the effects of four different lactate salts, namely sodium, lithium, potassium and magnesium lactate. Each series consisted of four fermentations which were permitted to go to completion as indicated by the titration of the acidity present. The only variable within each series was the concentration of the lactate salt being tested. The lactate ion buffer concentration ranged from 0.02 to 0.17 molar except when testing magnesium lactate which is less soluble. In this case the concentrations ranged from 0.0057 to 0.0153 molar. The mash used in each fermentation, regardless of the series, contained 30 gms of glucose and 30 gms of malt sprouts per liter, plus the required amounts of growth salts "A", "B", "C" and "D" as previously discussed. Experimental results of these fermentations are shown in Table III.

These results show that varying the concentrations of sodium, lithium, potassium and magnesium lactate do not have any appreciable effect upon the final acid lactate produced by Lactobacillus delbrueckii. Nevertheless, the results do show a definite buffer effect upon the final pH of the fermentations. This is very clearly demonstrated by the potassium lactate experiments. The lactate ion buffer concentration was increased from 1.78 to 13.0 gms/liter. The corresponding final pH values were pH 3.15 and pH 3.9; however, the final acid lactate concentration produced by the organism was practically the same in both cases.

TABLE III. EFFECT OF USING DIFFERENT LACTATE SALTS AS BUFFER AGENTS UPON THE FINAL ACID LACTATE PRODUCED BY *Lactobacillus delbrueckii* AT 37.5°C

Run No.	Lactate Ion Buffer, Grams/Liter	Final pH	Acid Lactate Fermented Grams/Liter	Fermentation Time Hours
SODIUM LACTATE BUFFER:				
31	3.75		11.95	120
32	6.38		12.42	120
33	9.50		13.20	120
34	15.30		12.50	120
LITHIUM LACTATE BUFFER:				
35	2.73		13.17	120
36	4.22		11.98	120
37	6.60		11.30	120
38	10.50		12.50	120
POTASSIUM LACTATE BUFFER:				
47	1.78	3.15	11.80	73.5
48	4.70	3.45	10.80	73.5
49	8.80	3.75	10.90	73.5
50	13.00	3.90	12.00	73.5
MAGNESIUM LACTATE BUFFER:				
51	0.51	3.10	10.87	73.5
52	0.67	3.10	10.43	73.5
53	1.05	3.16	10.95	73.5
54	1.53	3.14	11.37	73.5

Initial Volume: 1500 ml.
Glucose: 30 gms/liter
Malt Sprouts: 30 gms/liter

The magnesium lactate fermentations did not show such a marked buffer effect upon the final pH of the fermentation. This was due to the small changes in the lactate ion concentrations. Nevertheless, it will be observed from Table III that the pH was raised from pH 3.10 to pH 3.14 with the corresponding increase in the acid lactate from 10.87 gms/liter to 11.37 gms/liter.

The final pH of fermentations No. 31 to No. 38 inclusive, could not be measured because the pH meter being used at that time was acting in a very erratic manner. However, the results of experiments performed with sodium lactate at later dates corroborate the results obtained with potassium lactate, that is, sodium lactate does have a marked buffer effect upon the final pH of the fermentation. This is shown in Figure 8.

The experiments also demonstrate the fact that neither sodium, lithium, potassium or magnesium have a poisonous or inhibitory effect upon the metabolic activity of Lactobacillus delbrueckii at the concentrations used in these experiments.

Furthermore the results show that any one of the four lactate salts tested do meet the three specific requirements expected from a buffer agent discussed on page 36.

2. Effect of malt sprout concentrations

Two series of experiments were performed in order to determine the effect that the malt sprout concentration have upon the fermentation.

The first series, the results of which are shown in Figure 4, was performed under the conditions specified in Table VI for Runs No. 93-100 inclusive. The results show that the malt sprout concentration

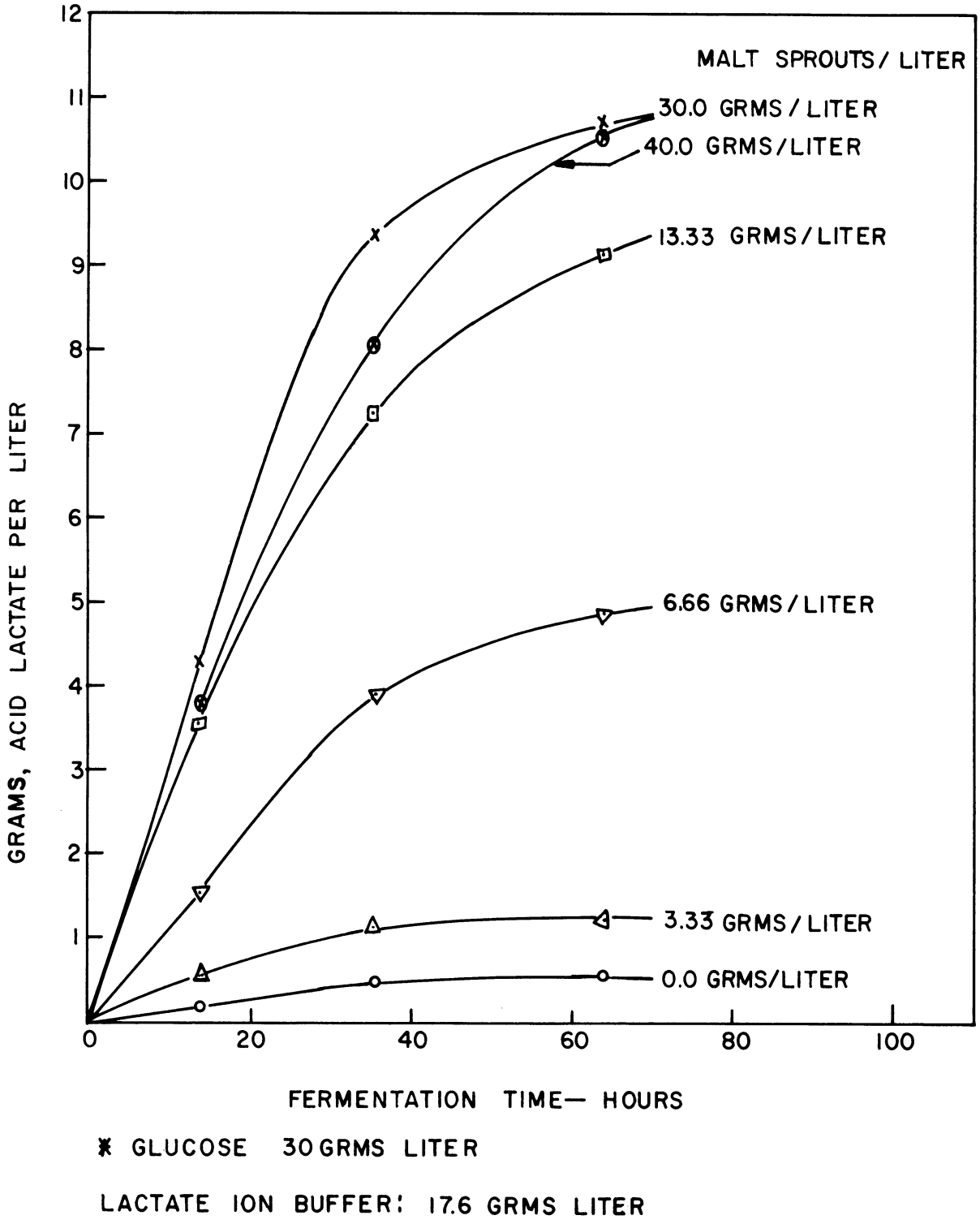


Figure 4. Effect of Varying the Malt Sprout Concentration upon the Acid Lactate Produced by Lactobacillus delbrueckii at 45.5°C in the Presence of Lactate Buffer*

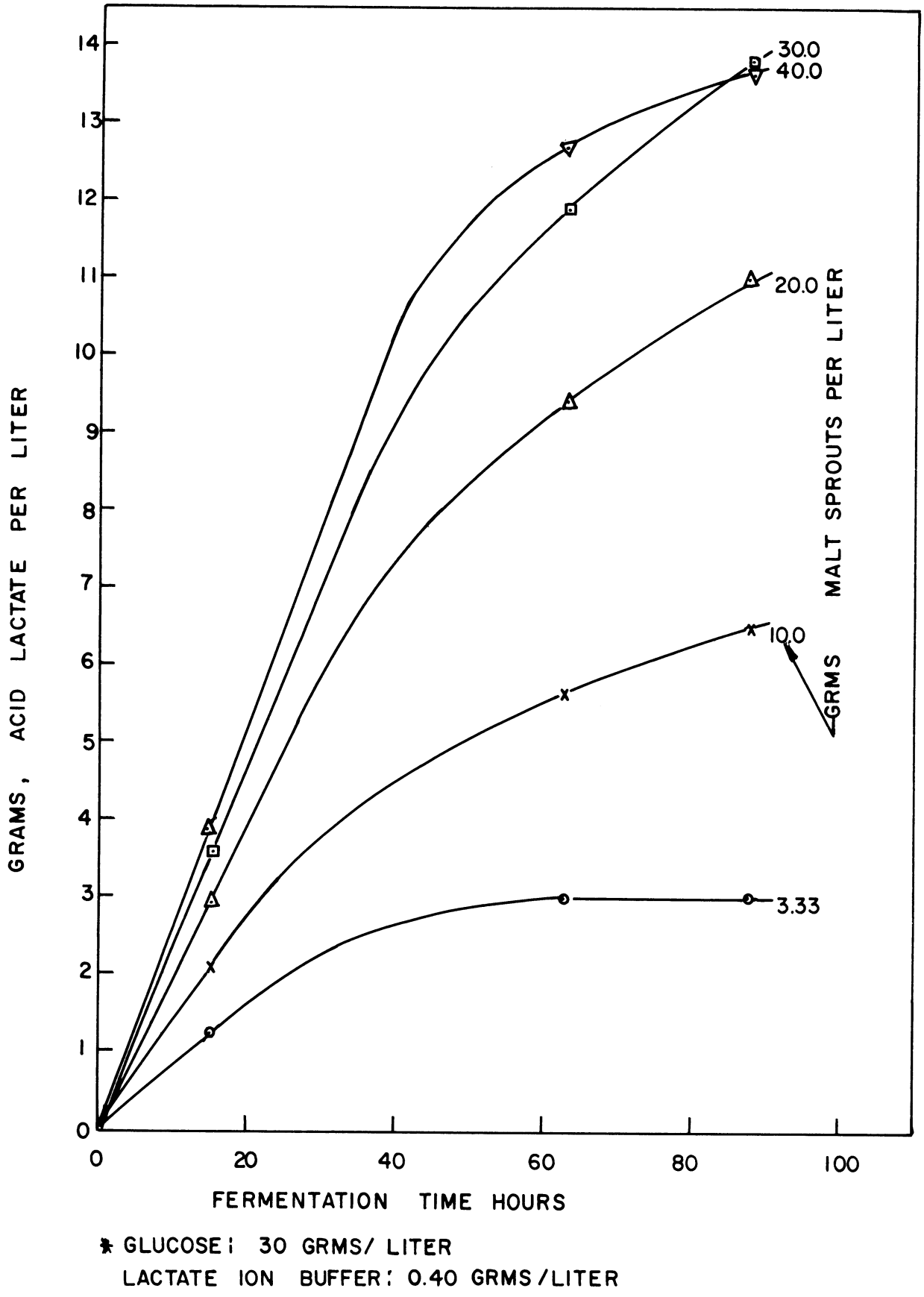


Figure 5. Effect of Varying the Malt Sprout Concentration upon the Acid Lactate Produced by Lactobacillus delbrueckii at 45.5°C in the Presence of Lactate Buffer*

has a definite effect upon the fermentation rate and upon the final acid lactate concentration. The effects are very noticeable up to malt sprout concentrations of 30-40 grams/liter. Larger concentrations failed to show any definite effects either on the fermentation rate or upon the final acid lactate concentration.

The second series, the results of which are shown in Figure 5, was performed under the conditions specified in Table VI for Runs No. 117-123 inclusive. This series represented an attempt to obtain some data on fermentations in which no lactate ion buffer was used. However, 45 grams of malt sprouts contained 0.47 grams of lactic acid. This acid was neutralized in order to adjust the original pH of each fermentation. Thus each fermentation actually contained lactate ion buffer in concentrations proportional to the malt sprouts used. Nevertheless, Figure 5 also shows the effect of malt sprout concentrations upon the fermentation rate and final acid lactate concentration. The conditions at which this series was performed are considerably different from the conditions of the first series. However, it also shows that a malt sprout concentration of 30-40 grams/liter seems to be the optimum with respect to the fermentation rate and also with respect to the final acid lactate concentration.

The effect of the malt sprout concentration upon the final pH of each fermentation within any one of the series is directly dependent upon the final acid lactate concentration obtained at different malt sprout concentrations. The lactate ion buffer concentration is practically constant within each series. Thus any variation in the final acid lactate concentration will also show itself in the final pH values shown in Table VI for Runs 93-100 and 117-123 inclusive.

3. Effect of substrate concentration

Four series of experiments were performed to determine whether the substrate concentration had any effect upon the fermentation course or the final acid lactate concentration.

Three of the four series were performed at the same concentration of malt sprouts, 30 grams/liter. Each series was performed at a different level of lactate ion buffer concentration. Glucose was used as the substrate in each of the four series.

The first series consisted of Runs No. 71-74 inclusive. It was the only one using a malt sprout concentration slightly higher than 30 grams/liter. The results of Run No. 71 seems to indicate a variation in the final acid lactate dependent upon the original glucose concentration. It should be noticed that there was not enough glucose originally present to permit the organism to produce the maximum acid lactate at that temperature. Runs No. 72, 73, and 74, which had more than enough glucose originally present for this purpose, do not show any appreciable variation in the final acid lactate concentration.

The second series consisted of Runs No. 101-104 and 137-143 inclusive. It also does not show any appreciable variation in the final acid lactate obtainable from the fermentations which originally contained more than 20 grams of glucose per liter. Figure 6, which shows the course of these fermentations, demonstrates also that there is no appreciable effect upon the fermentation rates due to variations in the glucose concentrations.

The third series consisted of Runs No. 109-116 inclusive. No appreciable difference in the final acid lactate is noticeable within this series from the fermentations which originally contained

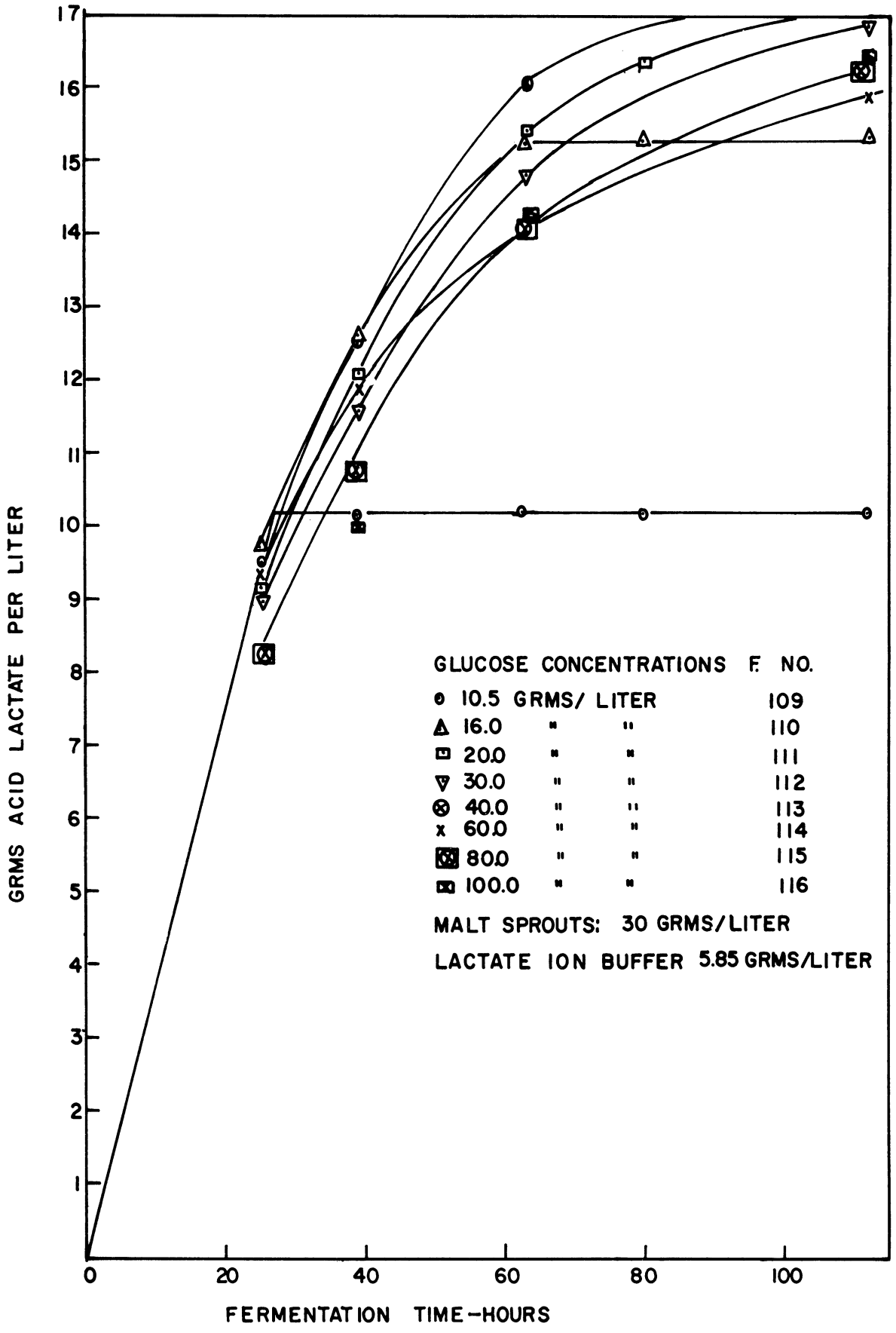


Figure 6. Effect of Varying the Glucose Concentration upon the Acid Lactate Produced by Lactobacillus delbrueckii at 43.5°C

more than 16 grams of glucose per liter. Similarly, there was no appreciable effect upon the fermentation rates in this series.

The fourth series consisted of Runs No. 124-130 inclusive. No appreciable difference in the final acid lactate and the fermentation rates were noticed within this series from the fermentations which originally contained more than 16 grams of glucose per liter.

These four series of fermentations demonstrate that the final acid lactate concentration and the fermentation rates are not dependent upon the initial glucose concentration as long as it is greater than 20 grams per liter.

4. Effect of fermentation temperature

Four fermentation temperatures were used during the course of the experimental program. An attempt to correlate the final acid lactate concentration with the fermentation temperature yielded Figure 7.

The acid lactate concentration representing a fermentation temperature of 37.5°C is the average value obtained from Runs No. 33 and 34 which would be the representative value of the final acid lactate obtainable under those specific conditions.

The acid lactate concentration representing a fermentation temperature of 40°C is the overall average value obtained from Runs 67-74 inclusive.

The acid lactate concentration representing a fermentation temperature of 43.5°C is the overall average value obtained from Runs 102-104 and 138-143 inclusive.

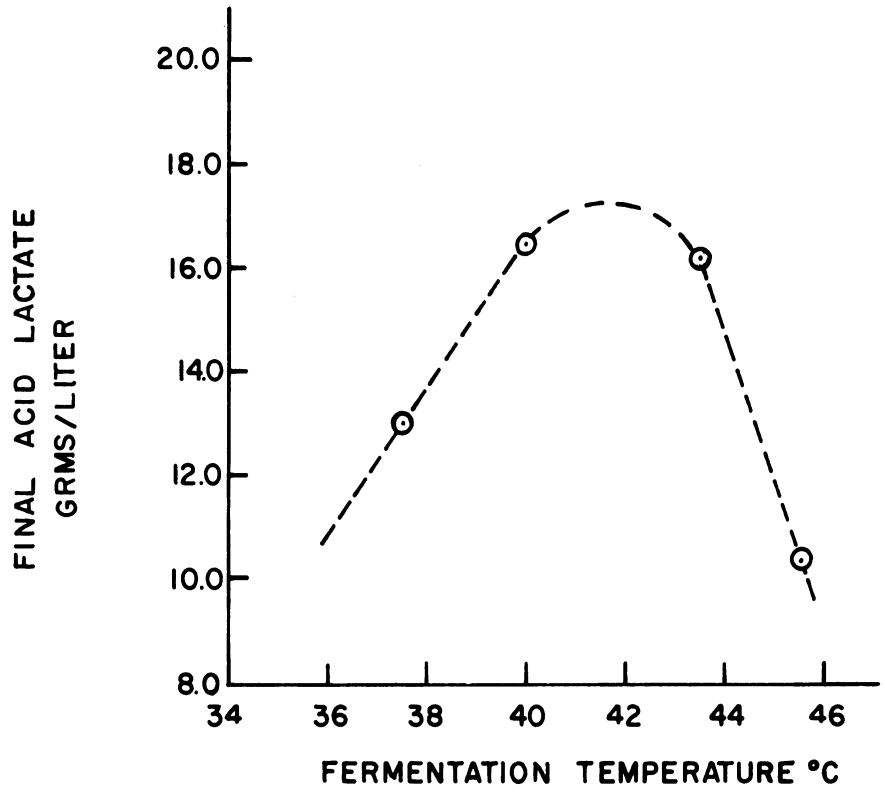


Figure 7. Effect of the Fermentation Temperature upon the Final Acid Lactate Produced by Lactobacillus delbrueckii

The acid lactate concentration representing a fermentation temperature of 45.5°C is the overall average value obtained from Runs 96-100 inclusive.

Figure 7 demonstrates the effect of the fermentation temperature upon the final acid lactate obtainable from fermentations buffered with sodium lactate corresponding to lactate ion buffer concentrations ranging from 10 to 19 grams per liter.

5. Effect of varying the lactate ion buffer concentration

Figure 8 shows typical fermentation curves for fermentations performed at various levels of lactate ion buffer concentrations where sodium lactate was used as the buffer agent.

The curves representing a lactate ion buffer concentration of 17.6 grams per liter were obtained by averaging the results of Runs 103-104 and 139-142 inclusive. These runs were used because they represent a constant malt sprout concentration of 30 grams per liter. Even though the glucose concentration varies from 20-60 grams per liter, the variations of the acid lactate and pH values during the course and at the end of the fermentations were almost negligible.

The curves representing a lactate ion buffer concentration of 5.85 grams per liter were obtained by averaging the results of Runs 110-116 inclusive. These runs were also performed at a constant malt sprout concentration of 30 grams per liter. The glucose was varied from 16 to 100 grams per liter. The variations of the acid lactate and the pH values during the course and at the end of the fermentations were also almost negligible.

The curves representing a lactate ion buffer concentration of 0.30 grams per liter were obtained by averaging the results of Runs

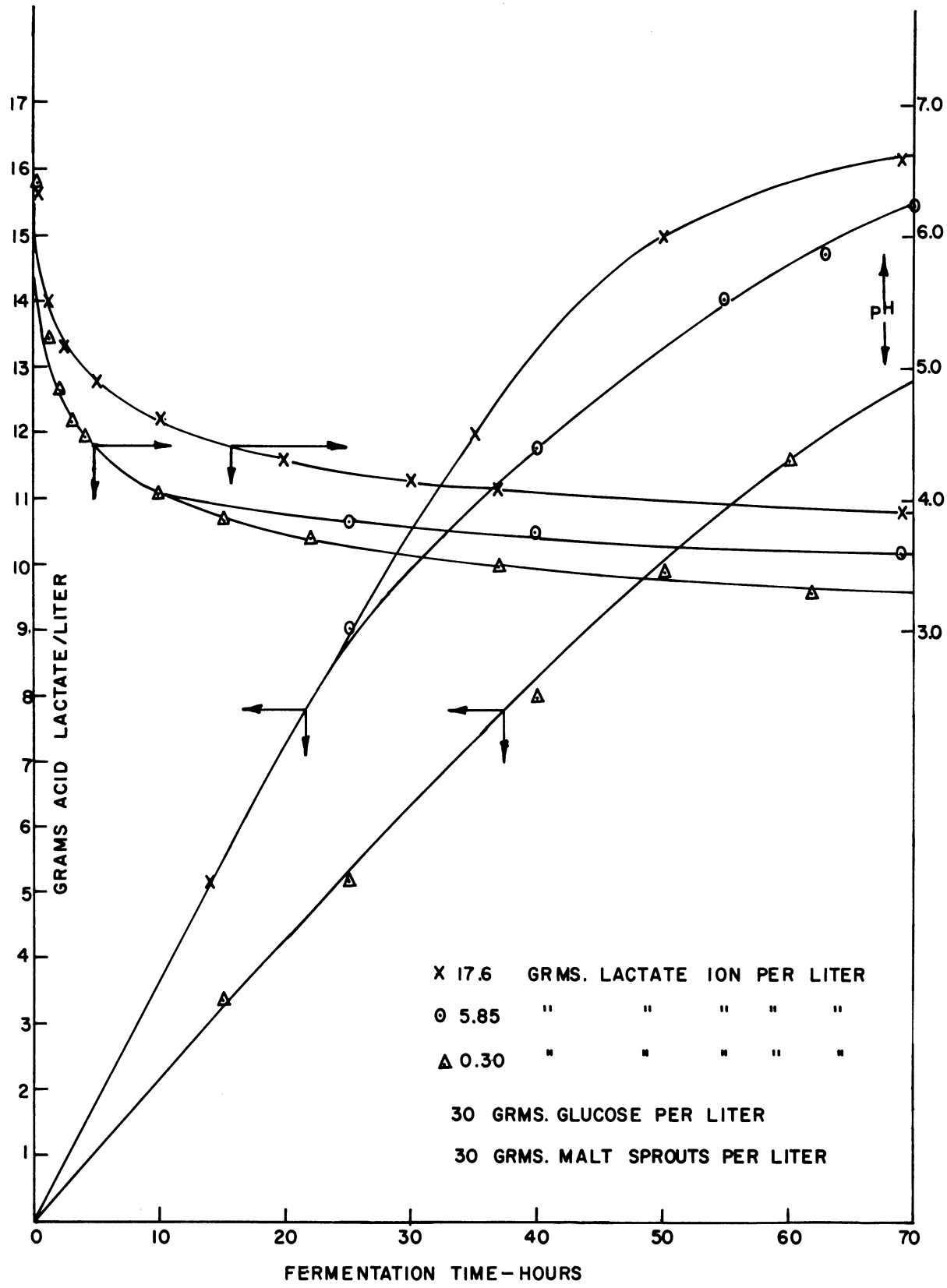


Figure 8. Effect of Varying the Lactate Ion Buffer Concentration upon the Acid Lactate Produced by Lactobacillus delbrueckii at a Fermentation Temperature of 43.5°C

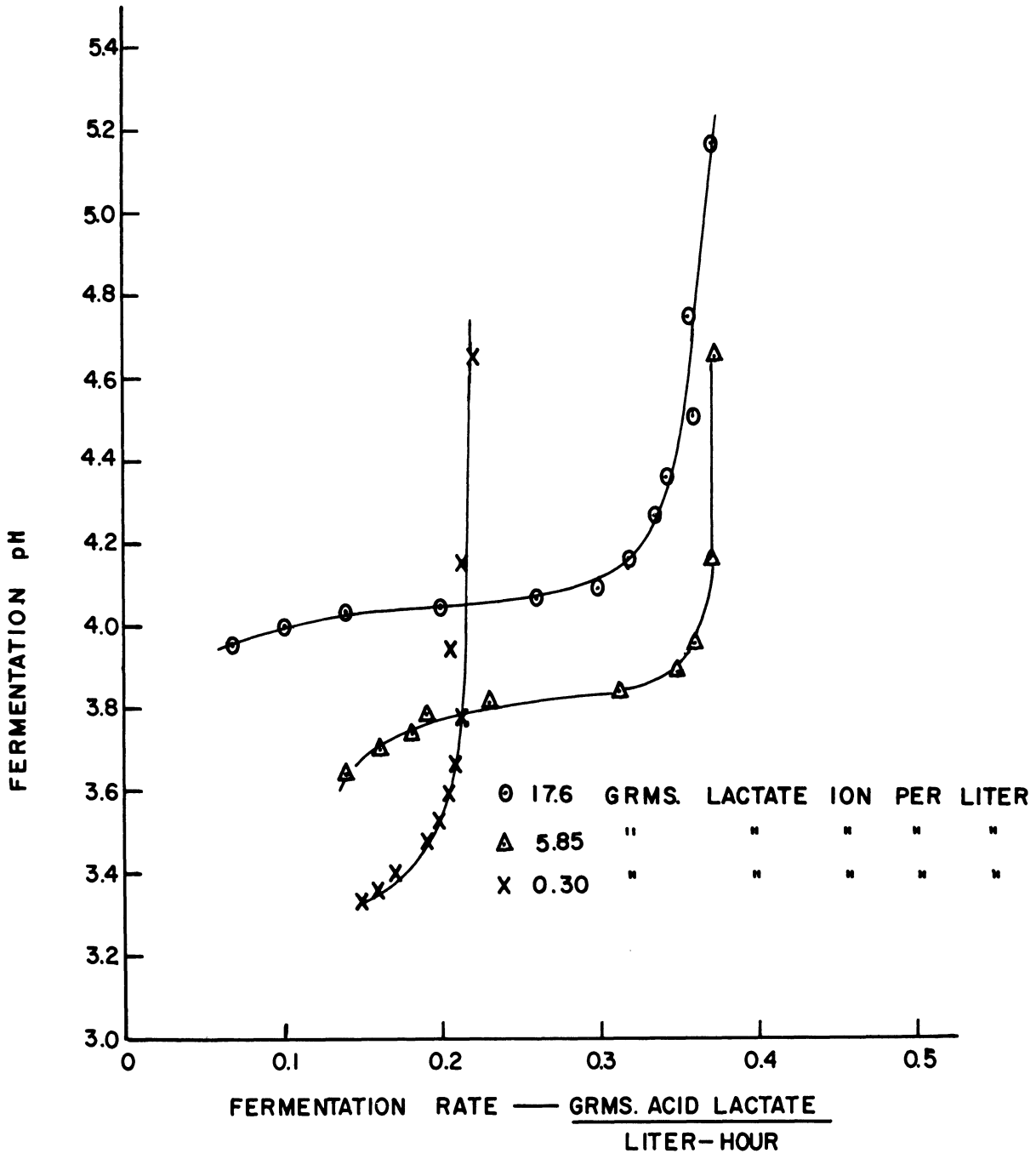


Figure 9. Fermentation Rates at Different Lactate Ion Buffer Concentrations as a Function of the pH at a Fermentation Temperature of 43.5°C

125-130 inclusive. These runs were also performed at a constant malt sprout concentration of 30 grams per liter. The glucose was varied from 16 to 80 grams per liter. The variations of the acid lactate and the pH values during the course and at the end of the fermentations were also negligible. The lactate ion buffer concentration of 0.30 grams per liter was obtained from the malt sprouts which contained 0.47 grams of acid lactate per 45 grams of malt sprouts. This acid was neutralized with sodium hydroxide when adjusting the original pH of each fermentation.

Figure 8 illustrates how the fermentations progress with respect to time, and of how the pH and acid lactate concentration are related to each other for a specific lactate ion buffer concentration. For example at a pH 4.0 the following is obtainable:

Lactate Ion Buffer	Acid Lactate Fermented	Fermentation Rates
Grams / Liter	Grams / Liter	Grams Acid Lactate / Liter-Hour
17.6	15.0	0.10
5.85	4.35	0.365
0.30	2.33	0.210

The fermentation rates were obtained by differentiating each curve graphically.

6. Inhibition effect of n-butyl alcohol upon the final acid lactate

Three series of experiments were performed in order to determine the inhibition effect of n-butyl alcohol upon the final acid lactate concentration. Each series consisted of four fermentations one

of which was used as control for each series. The only difference within each series was the amount of n-butyl alcohol added to each fermentation; the only difference among the series was the fermentation time at which the alcohol was added to the fermentations of each series. The results are shown in Table IV.

Referring to Table IV, the first series consisted of Runs 55-58 inclusive. Run 55 served as the control, that is, no n-butyl alcohol was added to it in order to compare the final acid lactate obtained with the other three fermentations of the series. Varying amounts of n-butyl alcohol were added to Runs 56,57, and 58 at the end of 48:40 fermentation hours and the fermentations were permitted to continue for 95 hours. The results show that the alcohol was added too late during the fermentation since the final acid lactate of the control was practically the same as that found in the other three fermentations. That is, the fermentations were complete when the alcohol was added.

The second series consisted of Runs 59-62 inclusive. Run 59 served as control. The alcohol was added at the end of 20:30 fermentation hours and the fermentations were permitted to continue for 68 hours. The results demonstrate the inhibiting effect of n-butyl alcohol upon the final acid lactate concentration.

The third series consisted of Runs 63-66 inclusive. Run 63 served as control. The alcohol was added immediately after inoculation of the fermentations and they were permitted to continue for 71 hours. The results also demonstrate the inhibiting effect of n-butyl alcohol upon the final acid lactate concentration.

TABLE IV. INHIBITION EFFECT OF normal-BUTYL ALCOHOL UPON THE FINAL ACID LACTATE PRODUCED BY *Lactobacillus delbrueckii* AT 37.5°C. THE ALCOHOL BEING ADDED AT DIFFERENT TIMES DURING THE FERMENTATIONS*

Run No.	Initial Volume ml.	Lactate Ion Buffer Grams Liter	Final Lactate Ion Grams Liter	Final pH	Acid Lactate Fermented Grams Liter	Alcohol Added ml.	Time Added After Inoc. Hrs.	Fermentation Time Hrs.
SODIUM LACTATE BUFFER:								
55	1620	17.6	28.7	3.65	11.10	0	Control	95
56	1625	17.6	29.3	3.60	11.70	15	48:40	95
57	1625	17.6	28.6	3.64	11.00	30	48:40	95
58	1625	17.6	28.8	3.64	11.20	45	48:40	95
59	1700	16.5	30.25	3.59	13.75	0	Control	68
60	1700	16.5	29.80	3.69	13.30	15	20:30	68
61	1700	16.5	21.75	4.10	5.25	30	20:30	68
62	1700	16.5	21.60	4.10	5.10	45	20:30	68
63	1500	18.7	32.20	3.50	13.50	0	Control	71
64	1500	18.7	29.25	3.65	10.50	15	00:00	71
65	1500	18.7	18.90	5.20	0.20	30	00:00	71
66	1500	18.7	18.00	5.60	0	45	00:00	71

* Glucose: 30 grams per liter.
Malt Sprouts: 30 grams per liter.

The percent inhibition, defined as follows

$$\frac{\text{Final Acid Lactate of Control - Final Acid Lactate with Alcohol}}{\text{Final Acid Lactate of Control}} \times 100$$

is shown graphically in Figure 10. The alcohol concentrations were used as parameters.

7. Inhibition effect of iso-amyl alcohol upon the acid lactate produced during the fermentations

Three series of experiments were performed in order to determine the inhibition effect of iso-amyl alcohol. The experiments consisted of ten fermentations. One was used as control and the remaining nine were divided into three series of three fermentations each. The only difference within each series was the amount of iso-amyl alcohol added to the fermentations. The only difference among each series was the fermentation time at which the iso-amyl alcohol was added. All of the fermentations were inoculated at the same time and were permitted to continue for 71.5 hours. Table V shows the results for each series at the end of 71.5 hours.

Figure 11 shows the effect of the iso-amyl alcohol upon the course of the fermentations. The graph shows in a very definite way what happens as soon as different amounts of iso-amyl alcohol are added to the fermentations. It shows that an alcohol concentration of 20 ml per liter of fermenting liquor will arrest the fermentation regardless of the time at which the alcohol is added.

The fermentations performed with 10 ml of alcohol per liter of fermenting liquor do show a very marked effect upon the acid lactate produced after the alcohol is added. When the alcohol is added at zero time, the fermentative bacteria are able to produce 3.9 grams of acid

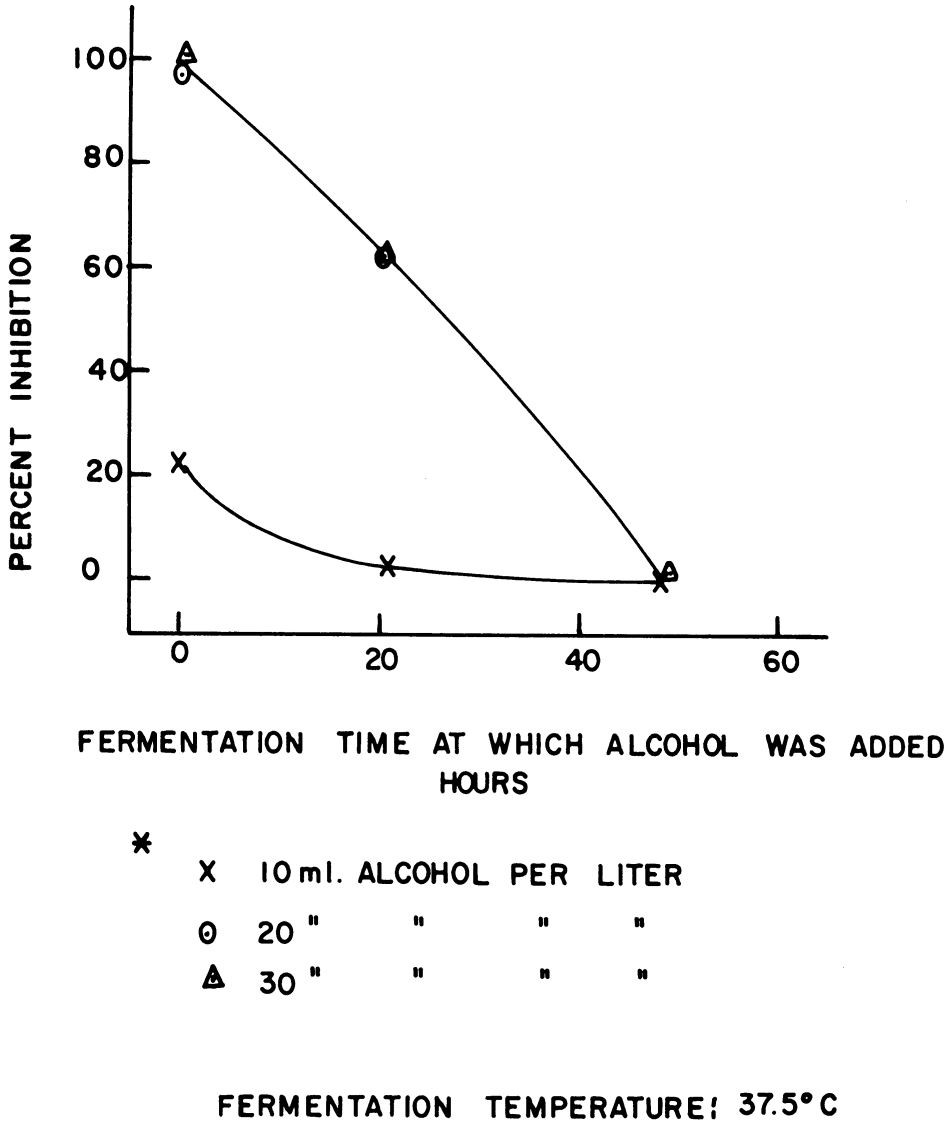


Figure 10. Inhibition Effect of n-Butyl Alcohol as a Function of the Time at which It Was Added During the Course of the Fermentation

TABLE V. INHIBITION EFFECT OF iso-AMYL ALCOHOL UPON THE FINAL ACID LACTATE PRODUCED BY *Lactobacillus delbrueckii* AT 40°C. THE ALCOHOL BEING ADDED AT DIFFERENT TIMES DURING THE FERMENTATION*

Run No.	Initial Volume ml.	Lactate Ion Buffer Grams/Liter	Final pH	Acid Lactate Fermented Grams/Liter	Alcohol Added ml.	Time Added After Inoc. Hrs.	Fermentation Time Hrs.
SODIUM LACTATE BUFFER:							
83	1500	17.6	3.9	16.8	None	Control	71.5
84	1500	17.6	4.59	3.96	15	0	71.5
85	1500	17.6	6.10	0	30	0	71.5
86	1500	17.6	6.10	0	45	0	71.5
87	1500	17.6	4.05	11.6	15	24	71.5
88	1500	17.6	4.23	8.38	30	24	71.5
89	1500	17.6	4.24	8.18	45	24	71.5
90	1500	17.6	3.99	13.9	15	50	71.5
91	1500	17.6	3.99	14.1	30	50	71.5
92	1500	17.6	3.99	14.6	45	50	71.5

* Glucose: 30 grams per liter
Malt Sprouts: 30 grams per liter

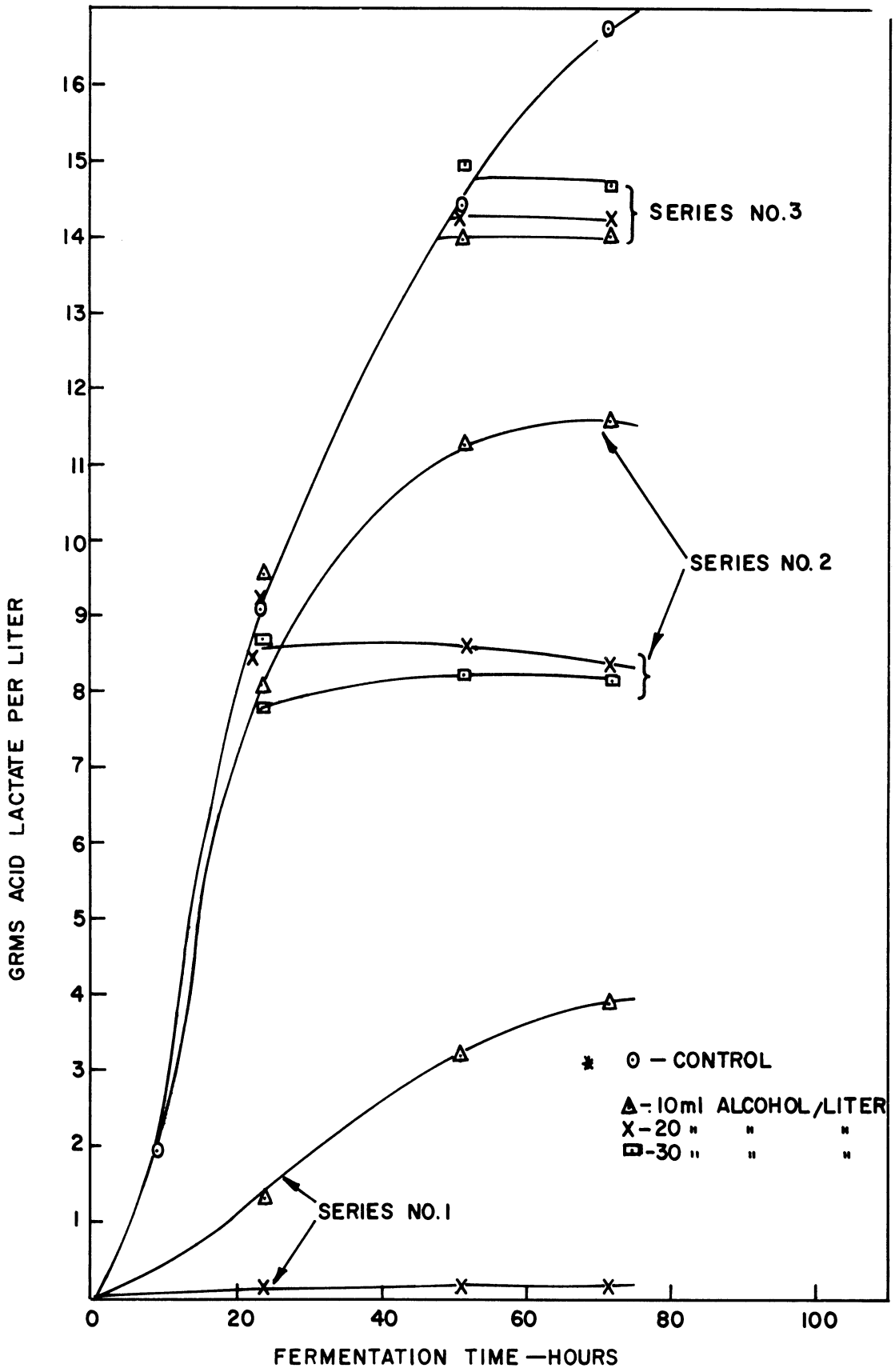


Figure 11. Effect of Adding Varying Amounts of iso-Amyl Alcohol* at Different Times after Inoculation at 40°C

lactate per liter; when the alcohol is added at the end of twenty-four hours the fermentative bacteria are still able to produce 3.6 more grams of acid lactate per liter. However, such is not the case when the alcohol is added at the end of fifty hours. In this case the fermentation is completely arrested by 10 ml of iso-amyl alcohol per liter of fermenting media. These facts demonstrate that the age of the culture has a definite effect upon the mode of action of the alcohol upon the organism. It also indicates that for a certain alcohol concentration, the speed at which a fermentation is arrested will depend upon the age of the fermentation. As long as the alcohol is added during the logarithmic growth phase of the organism, the fermentations will be arrested at the same rate.

The percent inhibition is shown graphically in Figure 12. The alcohol concentrations were used as parameters.

8. Adaptation of Lactobacillus delbrueckii

a. normal-Butyl alcohol: A considerable number of experiments were performed in an attempt to adapt Lactobacillus delbrueckii to the presence of normal-butyl alcohol.

One liter of the media used contained 30 grams glucose, 30 grams Difco yeast extract, 30 ml 60 percent sodium lactate solution, 2-1/2 ml of salt solution "A", 5 ml of each salt solutions "B", "C" and "D". The 30 ml of 60 percent sodium lactate solution contained 17.6 grams of lactate ion buffer. This media was divided into screw cap test tubes each containing 10 ml and the tubes were autoclaved at 121°C for 15 minutes.

An inoculum of 0.05 ml of the culture was used unless otherwise specified.

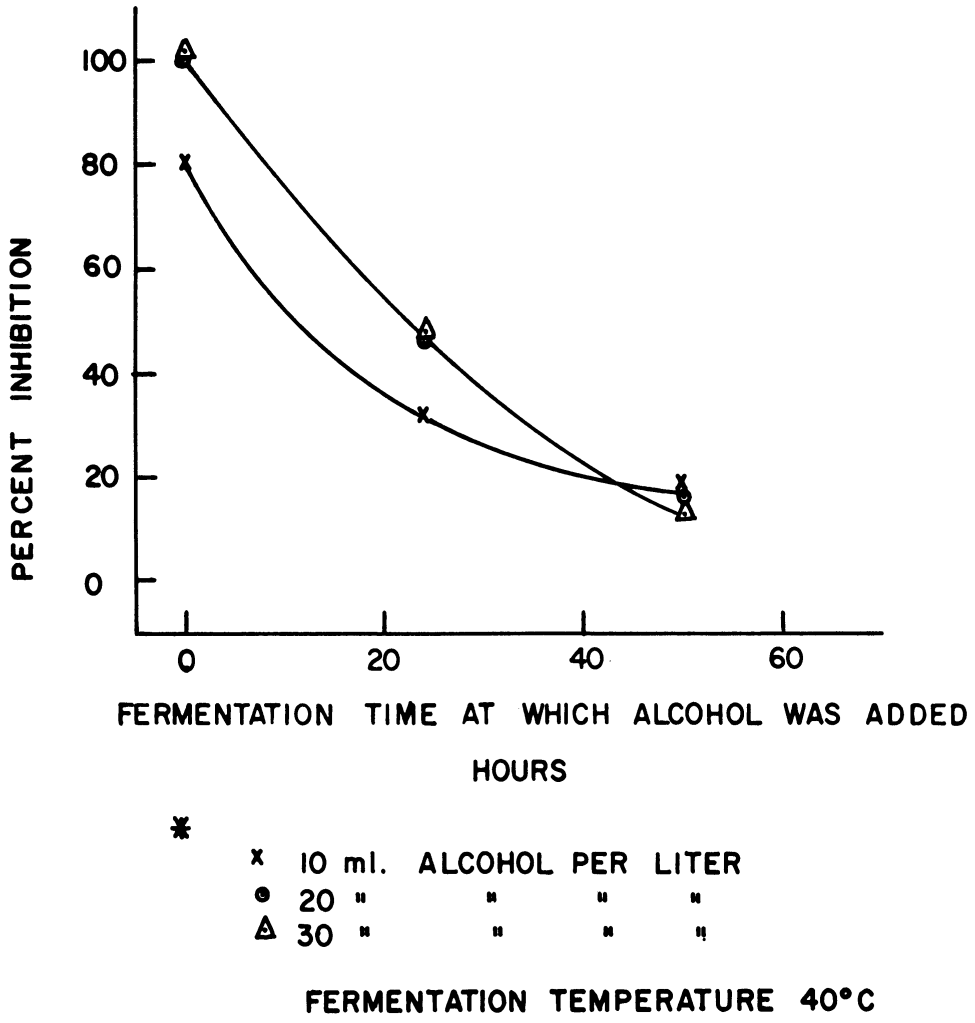


Figure 12. Inhibition Effect of iso-Amyl Alcohol as a Function of the Time at which It Was Added During the Course of the Fermentation*

TABLE VI. EFFECT OF VARYING THE LACTATE ION BUFFER, SUBSTRATE AND MALT SPROUT CONCENTRATIONS UPON THE FINAL ACID LACTATE PRODUCED BY Lactobacillus delbrueckii

Run No.	Initial Volume ml.	Lactate Ion Buffer Grams/Liter	Final pH	Acid Lactate Fermented Grams/Liter	Glucose Grams	Malt Sprouts Grams	Fermentation Time Hrs.	Fermentation Temp. °C
SODIUM LACTATE BUFFER:								
93	1500	17.6	6.00	0.506	45	0	64	45.5
94	1500	17.6	4.80	1.18	45	5	64	45.5
95	1500	17.6	3.68	4.86	45	10	64	45.5
96	1500	17.6	3.45	9.125	45	20	64	45.5
97	1500	17.6	3.59	9.10	45	30	64	45.5
98	1500	17.6	3.49	10.65	45	40	64	45.5
99	1500	17.6	3.59	9.86	45	50	64	45.5
100	1500	17.6	3.56	10.58	45	60	64	45.5
101	1500	17.6	4.06	10.26	15	45	72	43.5
102	1500	17.6	4.00	13.06	23	45	72	43.5
103	1500	17.6	3.94	14.50	30	45	72	43.5
104	1500	17.6	3.89	15.65	45	45	72	43.5
137	1500	17.6	4.13	10.20	15	45	69	43.5
138	1500	17.6	4.01	13.95	23	45	69	43.5
139	1500	17.6	3.90	16.44	30	45	69	43.5
140	1500	17.6	3.91	15.40	45	45	69	43.5
141	1500	17.6	3.89	16.50	60	45	69	43.5
142	1500	17.6	3.89	16.00	90	45	69	43.5
143	1500	17.6	3.99	12.81	120	45	69	43.5

TABLE VI (Continued)

Run No.	Initial Volume ml.	Lactate Ion Buffer Grams/Liter	Final pH	Acid Lactate Fermented Grams/Liter	Glucose Grams	Malt Sprouts Grams	Fermentation Time Hrs.	Fermentation Temp. °C
109	1500	5.85	3.8	10.46	17	45	63	43.5
110	1500	5.85	3.59	15.23	24	45	63	43.5
111	1500	5.85	3.61	15.40	30	45	63	43.5
112	1500	5.85	3.61	14.80	45	45	63	43.5
113	1500	5.85	3.58	16.03	60	45	63	43.5
114	1500	5.85	3.65	14.05	90	45	63	43.5
115	1500	5.85	3.63	14.10	120	45	63	43.5
116	1500	5.85	3.63	14.29	150	45	63	43.5
117	1500	0	6.40	0	45	0	88	43.5
118	1500	0.05	3.90	3.00	45	5	88	43.5
119	1500	0.15	3.50	6.50	45	15	88	43.5
120	1500	0.30	3.39	11.00	45	30	88	43.5
121	1500	0.47	3.35	13.80	45	45	88	43.5
122	1500	0.63	3.35	13.69	45	60	88	43.5
123	1500	0.78	3.35	15.98	45	75	88	43.5
124	1500	0.47	3.43	9.7	15	45	87	43.5
125	1500	0.47	3.31	14.2	23	45	87	43.5
126	1500	0.47	3.31	13.2	30	45	87	43.5
127	1500	0.47	3.31	13.25	45	45	87	43.5
128	1500	0.47	3.30	13.2	60	45	87	43.5
129	1500	0.47	3.30	12.9	90	45	87	43.5
130	1500	0.47	3.30	13.4	120	45	87	43.5

TABLE VI (Continued)

Run No.	Initial Volume ml.	Lactate Ion Buffer Grams / Liter	Final pH	Acid Lactate Fermented Grams / Liter	Substrate Grams	Malt Sprouts Grams	Fermentation Time Hrs.	Fermentation Temp. °C
SODIUM LACTATE BUFFER:					Sucrose			
27	1500	17.5		6.45	10	45	96	40
28	1500	17.5		9.40	20	45	96	40
29	1500	17.5		12.70	30	45	96	40
30	1500	17.5		13.70	45	45	96	40
14	1500	16.2		16.80	75	25	96	40
					Glucose			
13	1500	16.2		14.10	75	25	96	40
67	1500	19.0	3.9	12.00	45	25	96	40
68	1500	18.64	3.7	18.46	45	50	96	40
69	1500	19.33	3.8	15.87	45	75	96	40
70	1500	19.70	3.75	17.10	45	100	96	40
71	1500	14.90	3.83	14.10	25	50	96	40
72	1500	14.10	3.69	19.50	50	50	96	40
73	1500	14.65	3.69	18.15	75	50	96	40
74	1500	14.55	3.70	17.35	100	50	96	40
MIXED LACTATE SALTS: LACTATE ION FROM SODIUM LACTATE: 19.3 Grams per Liter MAGNESIUM LACTATE								
75	1500	0	3.78	19.60	45	45	124	40
76	1500	0.70	3.81	20.10	45	45	124	40
77	1500	2.40	3.80	18.80	45	45	124	40

The adaptation experimental procedure was as follows:

The stock culture was inoculated into a culture tube to which 0.2 ml of reagent grade n-butyl alcohol were then added. Twenty-four hours later this culture in alcohol was transferred to five culture tubes to which 0.2, 0.3, 0.4, 0.5 and 0.6 ml of n-butyl alcohol were added afterward. All of these cultures showed thick growths at the end of twenty-four hours, at which time each one of them was again transferred to the same alcohol concentrations. This procedure was repeated five times.

It was noticed that the amount of growth obtainable started diminishing usually after the third transfer. The growth obtained twenty-four hours after the fifth transfer was so slight that for all practical purposes it could be said that the organism had stopped growing. These results were repeatedly obtained.

The conditions investigated included a temperature of 37.5°C and a lactate ion buffer concentration of 17.6 grams per liter.

b. iso-Amyl alcohol: The adaptation experiments performed with reagent grade iso-amyl alcohol were of substantially the same nature as those with n-butyl alcohol. The media used was the same as the one used with the n-butyl alcohol experiments.

With iso-amyl alcohol complete growth inhibition was obtained after only the second or third transfer. Very often the growth after the second transfer was so sparse that for all practical purposes it could be said that the organism had stopped reproducing itself. However, the first transfer usually showed a very thick-stringy growth after twenty-four hours. The alcohol concentrations used were 0.1, 0.2, and 0.4 ml per 10 ml of media in the tubes.

These concentrations compare with those reported in Table V and Figure 11. Figure 11 suggests what might be happening during adaptation experiments of this sort.

Let us for a moment consider the experiments performed with 0.10 ml alcohol per 10 ml of media and base the analysis of the results upon Figure 11.

The first transfer of the stock culture to the alcohol media must have followed a fermentation curve very similar to the one representing Run 84. Twenty-four hours later the acid lactate concentration must have been approximately 1.5 grams per liter. The fermentative bacteria had been in direct contact with the alcohol for twenty-four hours.

When this culture was transferred to the same alcohol concentration, it must have been the equivalent of Run 85 which shows very little growth in Figure 11. This culture was transferred, once more, for the third time. To the organism the alcohol concentration must have been the equivalent to Run 86 which did not show any growth whatsoever.

Basing a conclusion upon the results of these adaptation experiments and the results shown in Figure 11, the author doubts whether Lactobacillus delbrueckii can be adapted to the saturation concentration of iso-amyl alcohol.

B. Extraction of the Acid Lactate

The fermented liquors were extracted with iso-amyl alcohol. The experiments were performed in order to determine the applicability to the case at hand of the equilibrium data published by Weiser (121) on the ternary system, iso-amyl alcohol - lactic acid - water.

Figure 13 shows Weiser's equilibrium data at 25°C, 40°C and 49.5°C in a right triangular plot. The curve representing the 40°C condition was obtained by the linear interpolation between the 25°C and the 49.5°C curves.

Figure 13 indicates that the portion of the diagram enclosing lactic acid mass fractions from 0 to 0.025 may be considered as straight lines. Figure 14 shows this section of the diagram drawn on an expanded scale for the lactic acid mass fraction ordinate.

Table VII shows the chemical compositions of the fermented liquors that were extracted. Liquors No. 6, 7, and 8 did not receive any purification other than filtration through a piece of clean, white cotton cloth to remove the malt sprout solids. Every extraction experiment performed on these three liquors was done in the presence of all the fermentation products. No attempt was made to remove any of the impurities such as residual glucose, lactate ion buffer, or bacteria prior to the extractions.

The extract phases obtained from liquors No. 6, 7, and 8 were water clear. Nevertheless, a coagulum formed throughout the extract phase which was easily agglomerated by gentle swirling. In every experiment the coagulum settled to the bottom of the extract phase thus accumulating at the interface between the extract phase and the solvent. Visual inspection of the raffinate phases showed that the cell population had been considerably diminished, indicating that the iso-amyl alcohol caused coagulation of the bacterial cells.

Chemical analysis of the extract phases demonstrated that the solvent did not extract sufficient glucose or lactate ion buffer to be determined by the analyses used in this work.

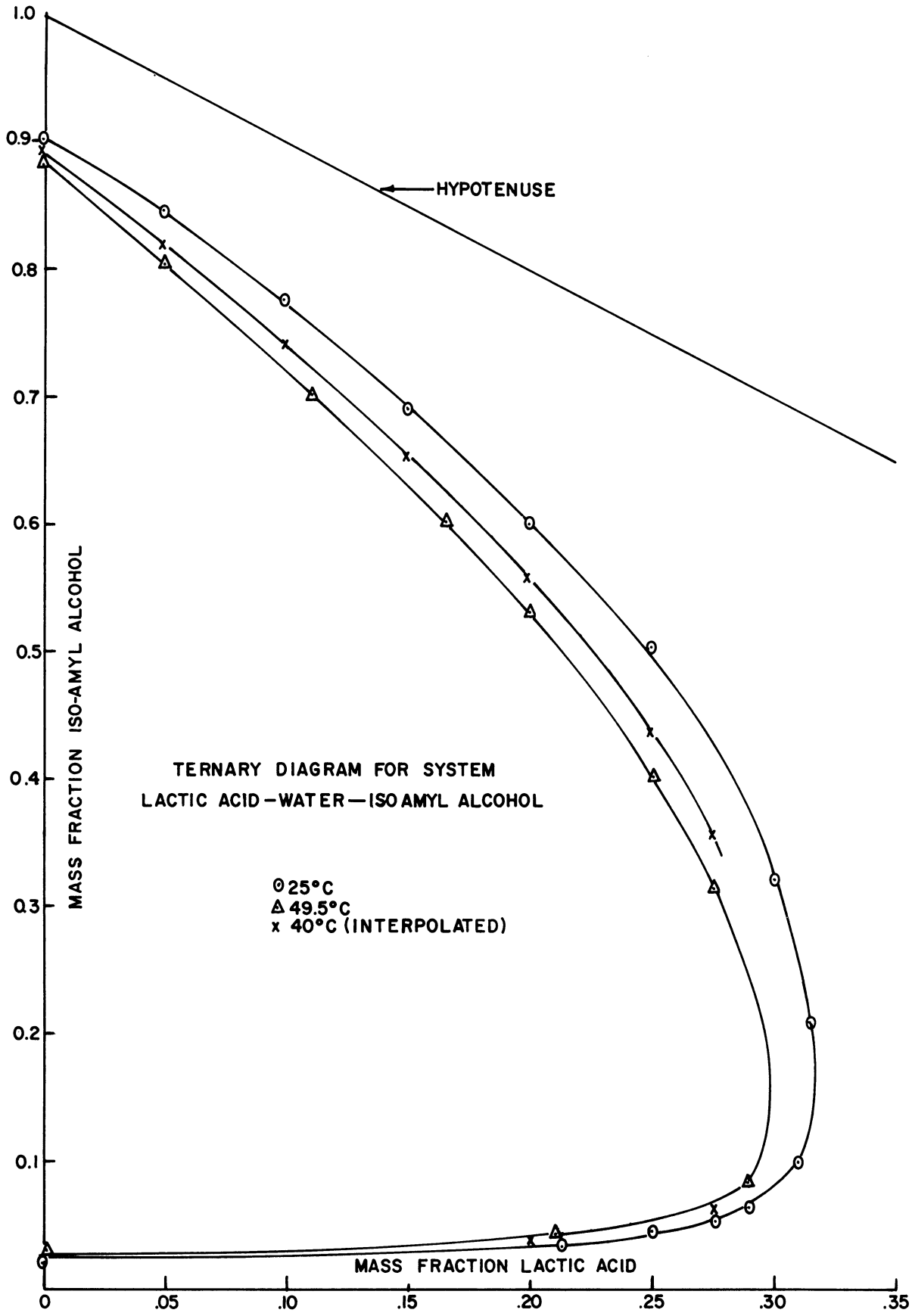


Figure 13. Ternary Diagram for System, Lactic Acid - Water - iso-Amyl Alcohol

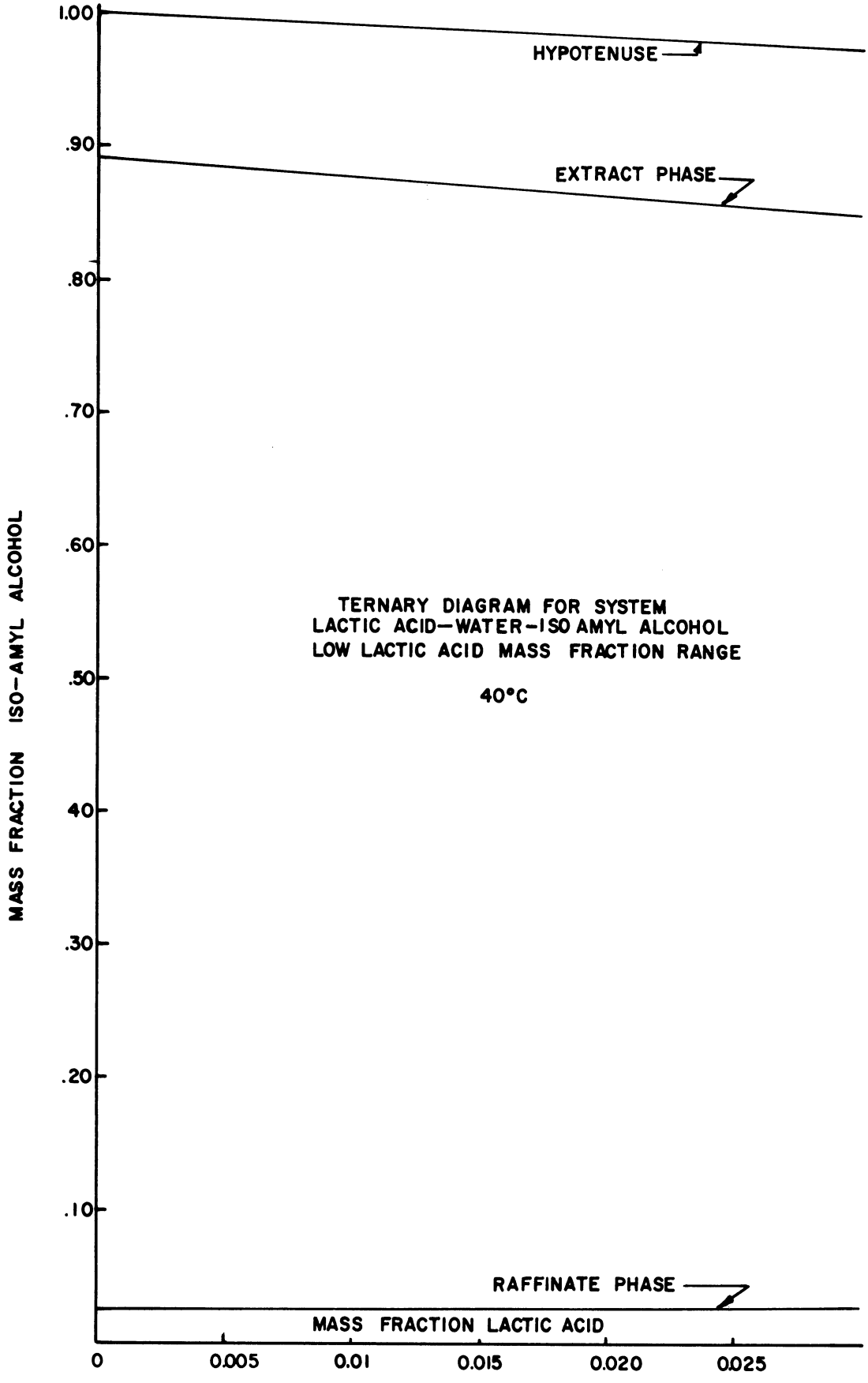


Figure 14. Ternary Diagram for System, Lactic Acid - Water - iso-Amyl Alcohol. Low Lactic Acid Mass Fraction Range 40°C

TABLE VII. CHEMICAL COMPOSITION OF THE LACTIC ACID FERMENTATION LIQUORS EXTRACTED WITH iso-AMYL ALCOHOL AT 40°C

Liquor No.	Glucose Grams/Liter	Lactate Ion Buffer Grams/Liter	Lactic Acid Grams/Liter	Lactate Salt Used	Specific Gravity	Lactic Acid Mass Fraction x 10^3
6	5.95	1.96	12.40	Sodium	1.015	12.20
7	4.96	17.6	11.69	Sodium	1.020	11.45
8	5.56	4.61	11.30	Calcium	1.015	11.15
98	17.80	17.6	11.05	Sodium	1.023	10.80
98-c	47.40	46.8	29.40	Sodium	1.049	28.50

The lactate ion buffer contained in liquor No. 8 consisted of calcium lactate. The extract phases were tested particularly for the calcium ion. However, treatment with oxalic acid failed to show the presence of calcium in the extract phase.

Extraction liquor No. 98 was obtained from fermentation No. 98. It was clarified by passage through a positive pressure-Seitz filter. Liquor No. 98-c was obtained by concentrating liquor No. 98. This concentration was carried out in order to check the tie lines on the ternary diagram at higher acid-lactate concentrations. It was also necessary to determine whether a concentration operation performed at pH 3.5 would hydrolyze the unfermented glucose.

The extract obtained with liquor No. 98 was water clear and it did not even have traces of coagulation. However, the extract obtained with liquor No. 98-c did contain a brownish tinge showing that the concentration step must have hydrolyzed some of the sugar and some of the hydrolysis products had been extracted by iso-amyl alcohol.

Figure 16 shows the equilibrium tie lines obtained with liquor No. 6. It can be noticed that two of the tie lines coincide with the addition points J_2 and J_3. However the J_1 tie line is somewhat displaced.

Figure 17 shows the equilibrium tie lines obtained with liquor No. 7. In this instance it can be noticed that the three tie lines do coincide with the respective addition points J_1, J_2 and J_3.

Figure 18 shows the equilibrium tie lines obtained with liquor No. 8. The two tie lines indicate a very slight deviation from their corresponding addition points J_2 and J_3. The extraction corresponding to J_1 was ruined on account of a leaky stop-cock.

Figure 19 shows the equilibrium tie line obtained with liquor No. 98. The tie line indicates a perfect coincidence with addition point J.

Figure 20 shows the equilibrium tie line obtained with liquor No. 98-c. It indicates a nearly perfect coincidence with the addition point J.

All results of the extraction experiments are shown in Table VIII.

Figure 15 shows the equilibrium distribution of lactic acid between the extract and raffinate phases at different lactate ion buffer concentration.

The coincidence between the tie lines and the corresponding J points indicate that the extract and raffinate phases had reached equilibrium at the time they were analyzed. It also shows that the system meets condition "a" as specified on page 68.

Table VIII shows slight deviations between the experimental and graphical value of the extract to raffinate ratios. However, the deviations are small and it indicates that the system meets condition "b" as specified on page 68. The results shown in Table VIII plus the fact that the extracts obtained were water clear demonstrate that Weiser's (121) equilibrium data is applicable to our case; that is, to extract the fermented liquors without any previous purification.

Furthermore, Figure 21 shows the graphical solution for the extraction of liquor No. 7 when using Weiser's equilibrium data for the extraction of pure lactic acid-water solutions. The extract to raffinate ratios obtained graphically show a very good agreement with the graphical ones reported in Table VIII for liquor No. 7. This also indicates that Weiser's data definitely applies to the case at hand.

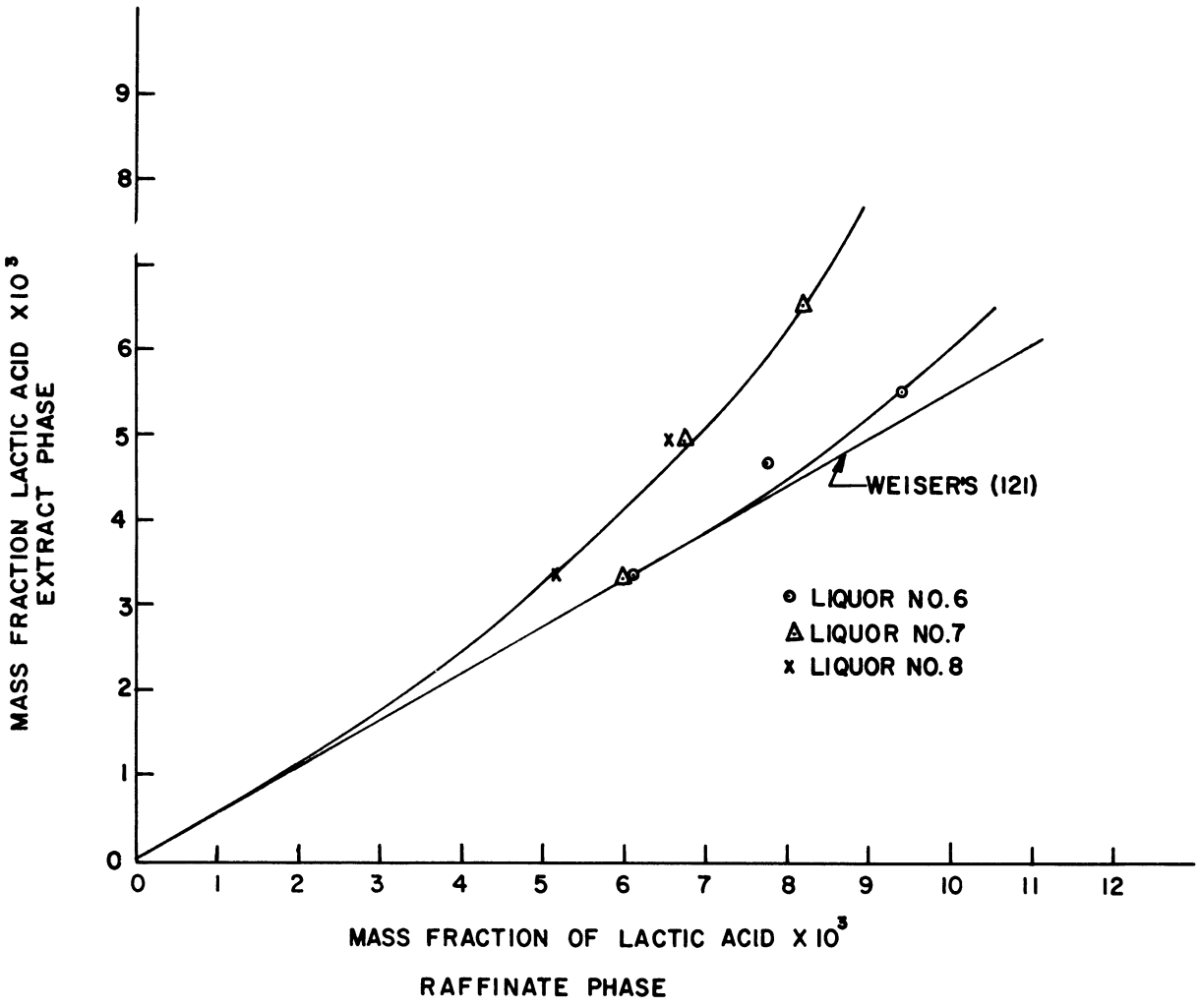


Figure 15. Equilibrium Distribution of Lactic Acid Between Liquor and iso-Amyl Alcohol when Extracting Fermentation Liquors at 40°C

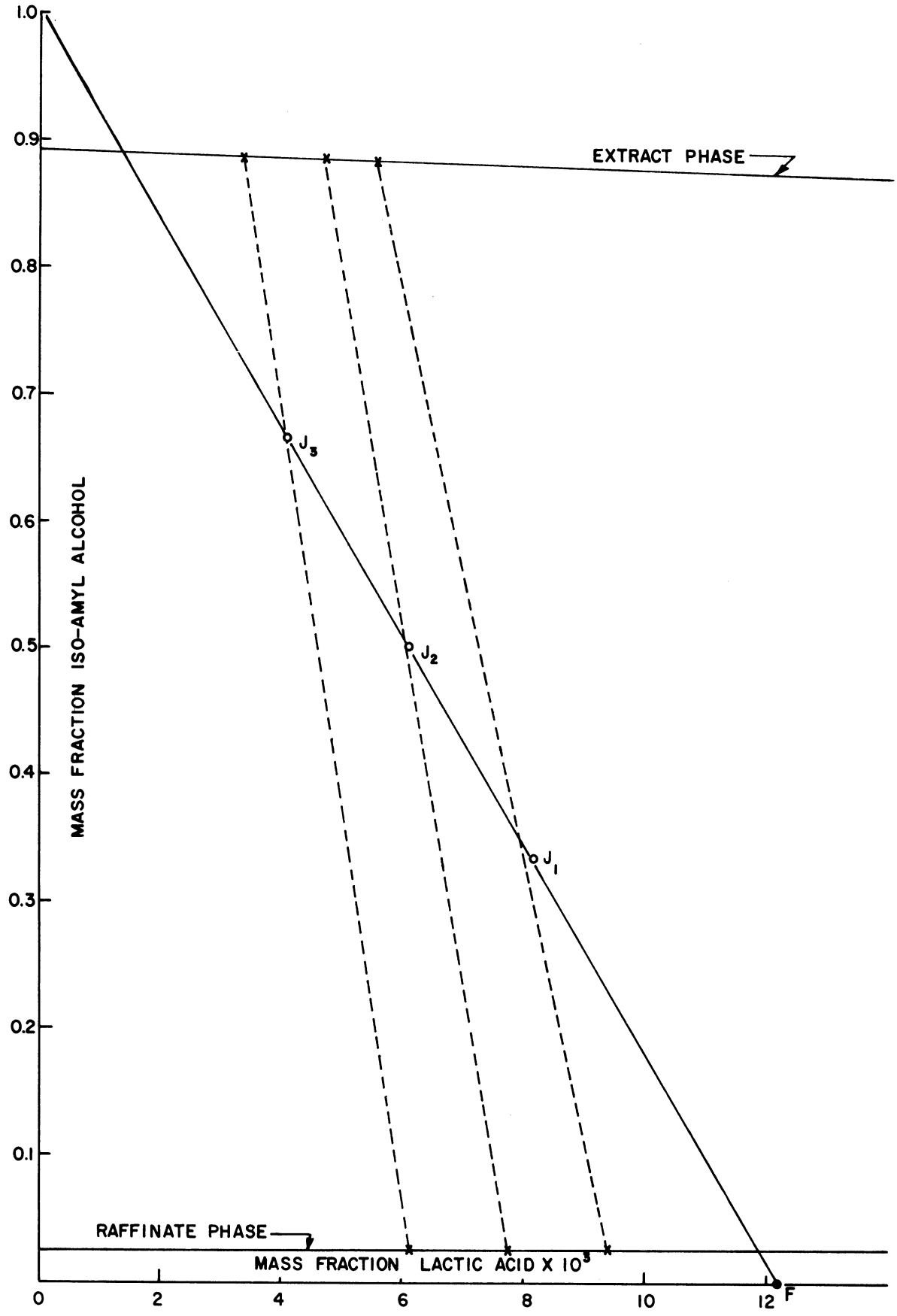


Figure 16. Extraction of Liquor No. 6

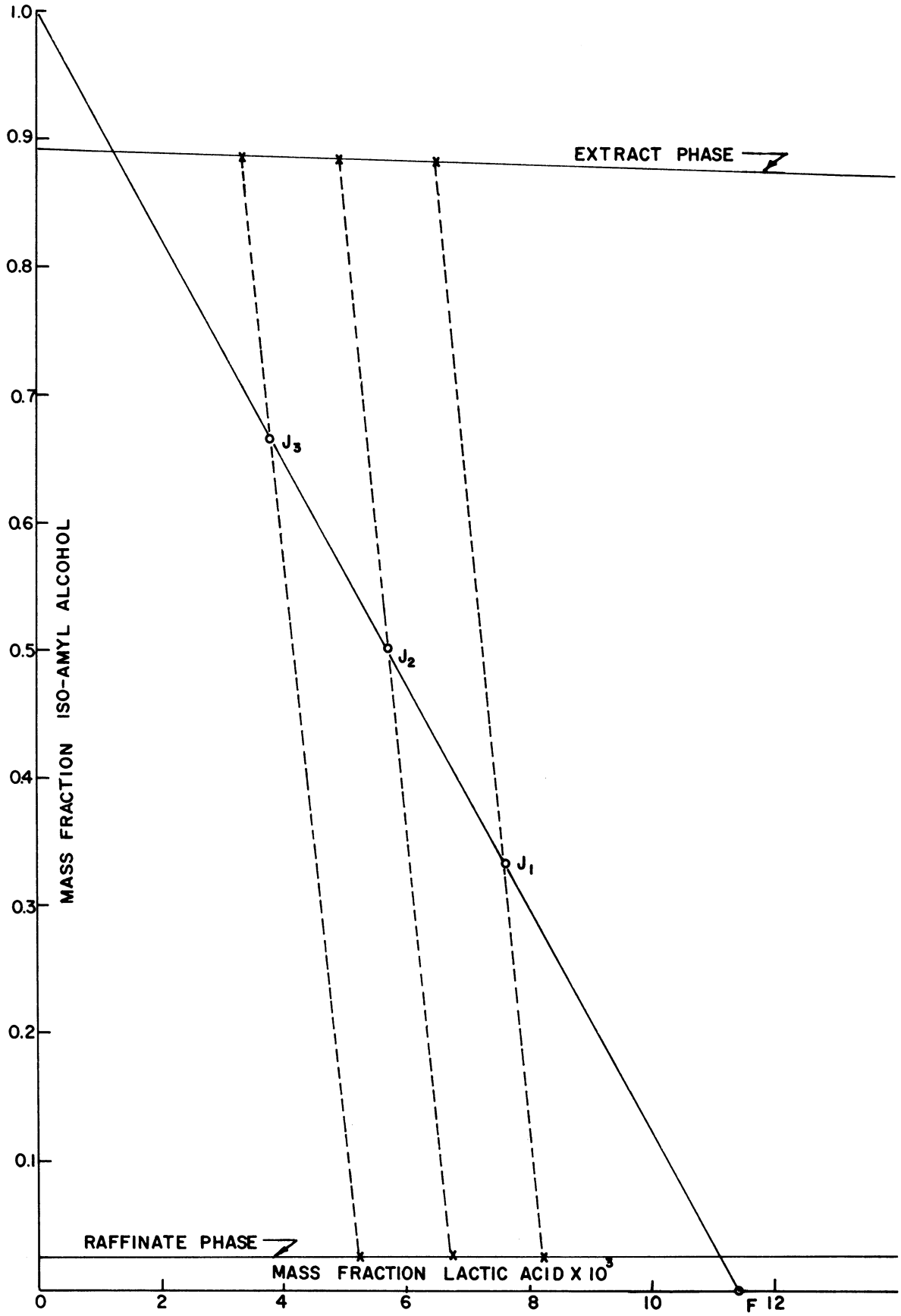


Figure 17. Extraction of Liquor No. 7

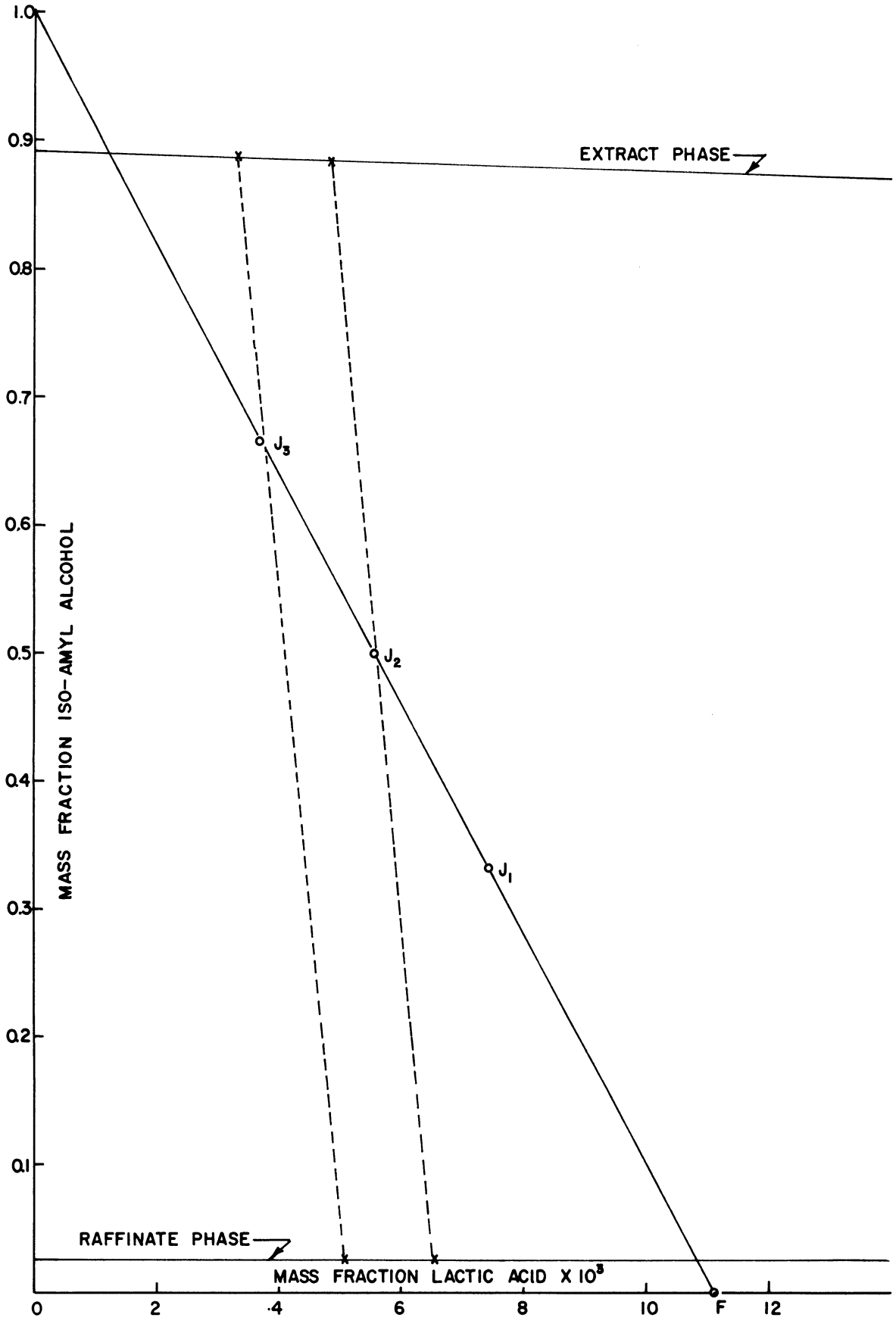


Figure 18. Extraction of Liquor No. 8

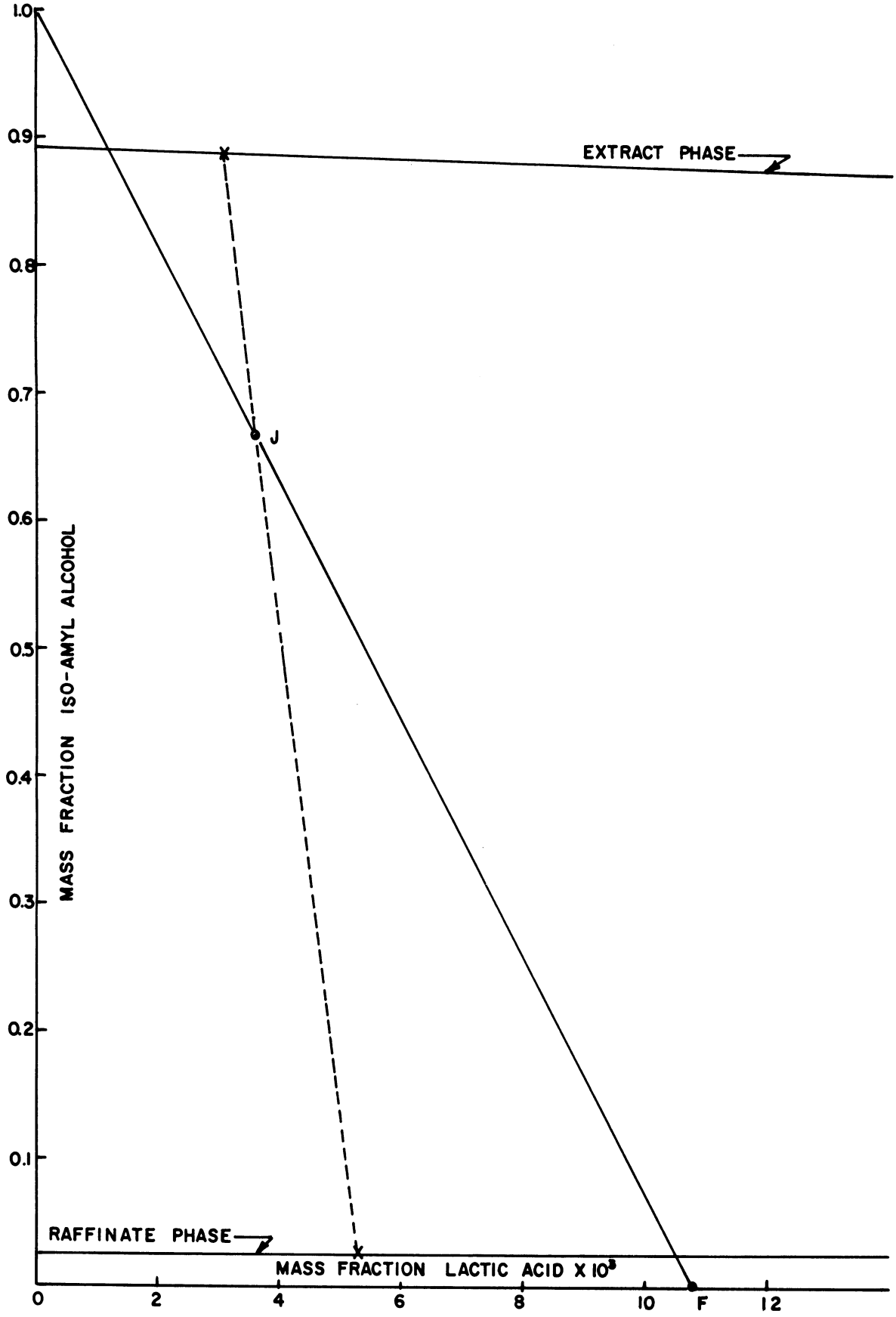


Figure 19. Extraction of Liquor No. 98

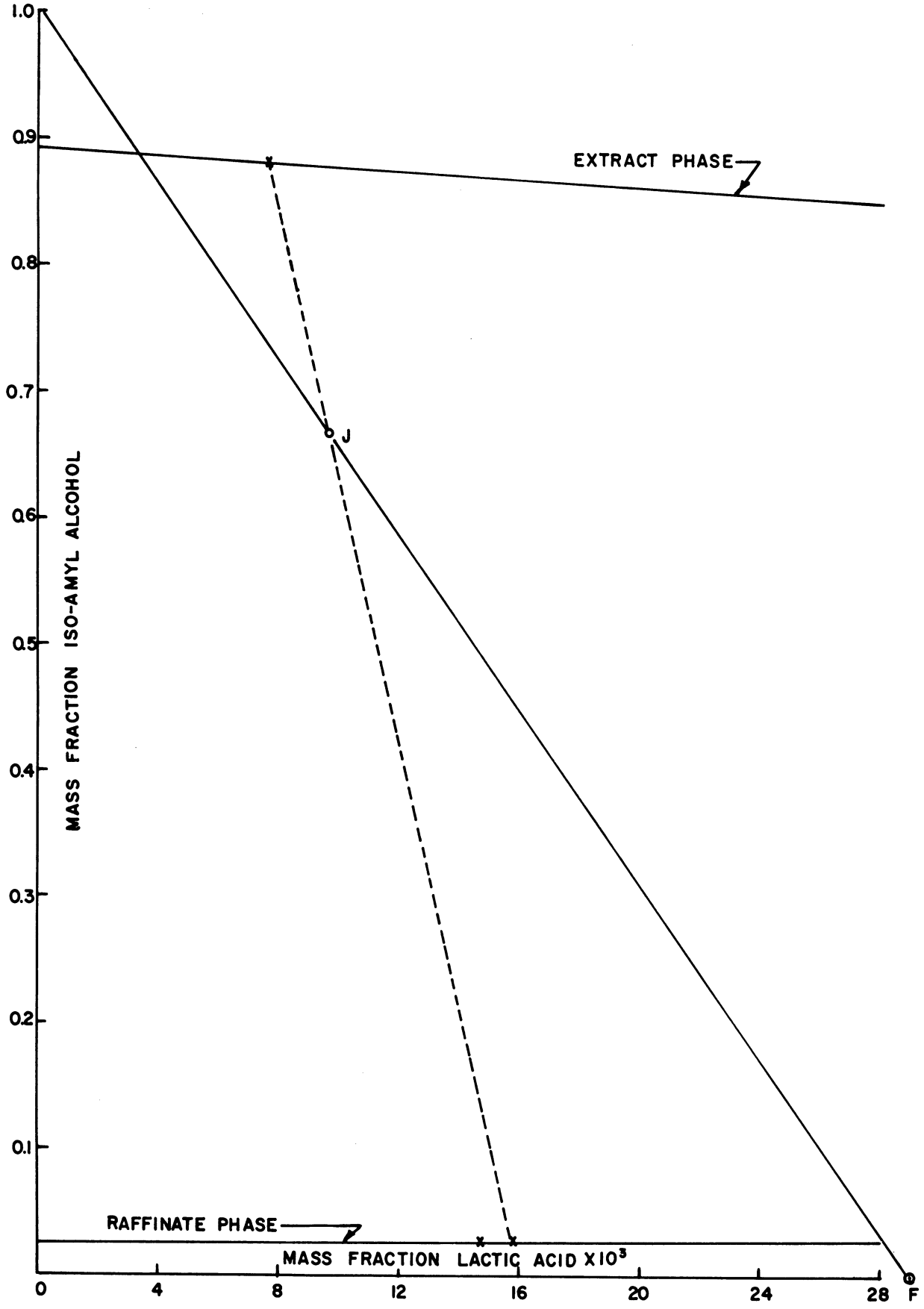


Figure 20. Extraction of Liquor No. 98-c

TABLE VIII. RESULTS OF THE EXTRACTION EXPERIMENTS

Feed		Solvent*	Extract		Raffinate				Extract / Raffinate		
Liquor No.	Grams	Grams	Mass Fraction HL x 10^3	Grams	Mass Fraction HL x 10^3	Grams	Sugar Grams / Liter	Lactate Ion Buffer Grams / Liter	Experimental	Graphical	$(HL)_E/(HL)_R$
6	100	50	5.55	58	9.4	92	6.47	1.96	0.631	0.603	0.591
	100	100	4.70	113	7.75	87	6.47	1.96	1.300	1.26	0.606
	100	200	3.35	226	6.15	74	7.36	2.09	3.055	2.92	0.545
7	100	50	6.53	58	8.2	92	5.4	17.6	0.631	0.556	0.795
	100	100	4.95	113	6.75	87	6.12	19.9	1.300	1.240	0.734
	100	200	3.36	228	5.25	72	6.24	20.3	3.165	2.900	0.640
8	100	100	4.95	113	6.50	87	6.4	5.3	1.30	1.15	0.755
	100	200	3.35	229	5.13	71	6.9	6.5	3.22	2.78	0.653
98	100	200	3.05	224	5.3	77			2.91	2.90	0.576
98-c	75	150	7.61	170	15.9	55			3.09	2.95	0.479

* iso-Amyl alcohol.

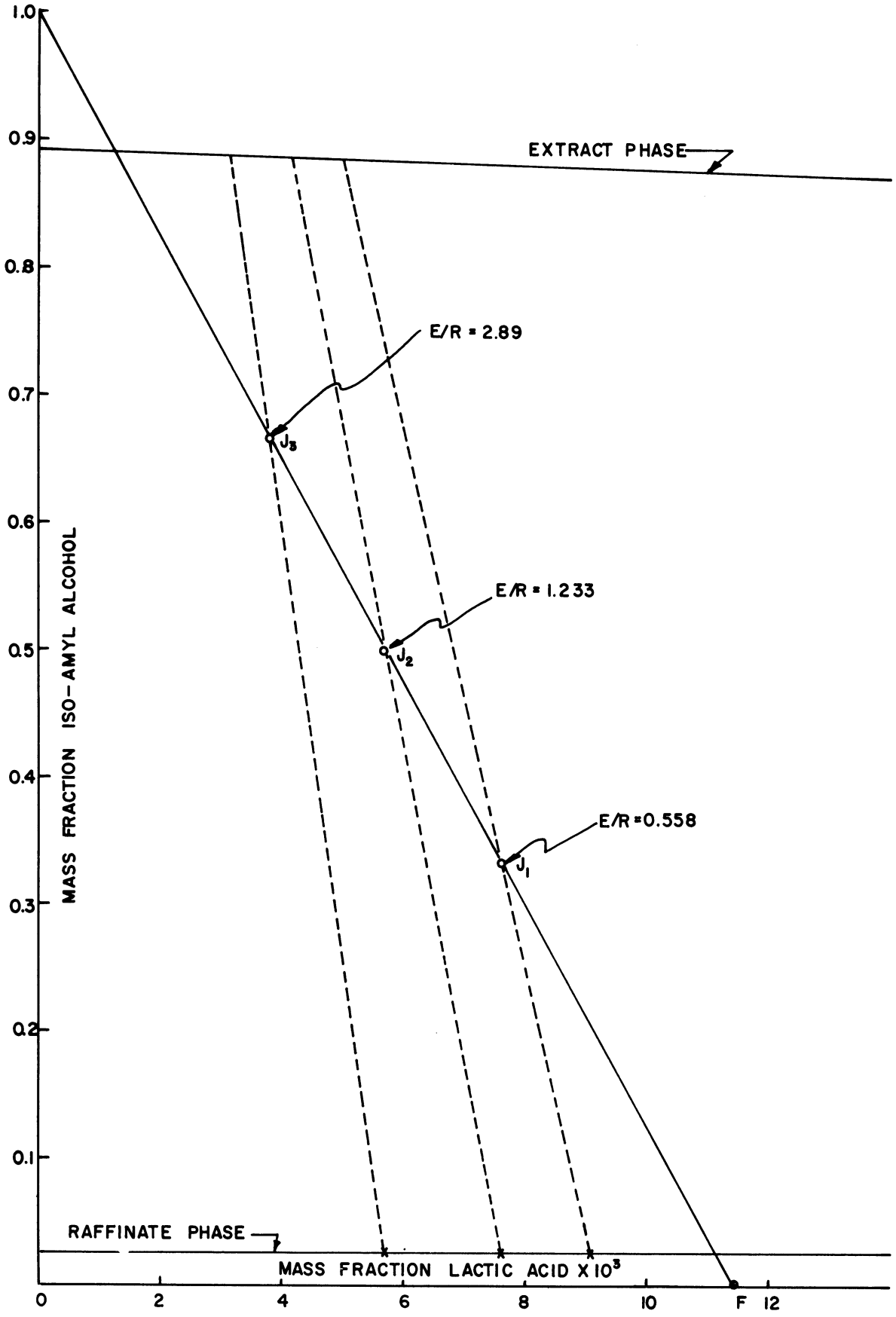


Figure 21. Graphical Solution for the Extraction of Liquor No. 7 Using Weiser's Pure Water Equilibrium Data at 40°C

C. Recirculation

A number of very specific experiments were performed while studying the recirculation phase of the experimental program.

The first experiment consisted of extracting a forty-eight hour fermentation. One liter of the fermented liquor contained the following: 18.8 grams of lactate ion buffer, 82 grams of glucose, 12.3 grams of acid lactate and all of the growth factors plus the impurities. Two 400 ml aliquots were extracted, one with 560 ml of n-butyl alcohol and the other with 600 ml of iso-amyl alcohol. Fifty ml samples of each of the raffinates were placed in the incubator and allowed to ferment for a second forty-eight hour period. Visual inspection and chemical analyses demonstrated that no further metabolic activity had taken place in either of the raffinate samples during the second forty-eight hour interval.

The second experiment consisted of extracting another forty-eight hour fermentation. One liter of the fermented liquor contained the following: 18.5 grams of lactate ion buffer, 55 grams of glucose, 10.5 grams of acid lactate and all of the growth factors plus the impurities. One liter was extracted with 1200 ml of iso-amyl alcohol. The raffinate was divided into three equal portions which were treated as follows:

First portion: Alcohol completely removed, made back to original volume, sterilized, reinoculated and incubated for forty-eight hours.

Second portion: Incubated as such without any further treatment.

Third portion: Reinoculated and incubated as such.

Visual inspection and chemical analyses of each portion at the end of forty-eight hours demonstrated that no further metabolic activity had taken place in either one of them.

The third experiment was of the same nature as the second one with the exception that n-butyl alcohol was used as solvent. The results obtained coincided with the ones of the previous experiment.

Another series of experiments consisted of extracting regular mashes with iso-amyl alcohol prior to being inoculated. The alcohol was completely removed from the raffinate by evaporation; the raffinate was then divided into two portions treated as follows:

First portion: Not refortified with malt sprouts, sterilized, inoculated and incubated for forty-eight hours.

Second portion: Refortified with malt sprouts, sterilized, inoculated and incubated for forty-eight hours.

Visual inspection and chemical analyses of both portions demonstrated that the raffinates of iso-amyl alcohol extractions need to be refortified after the alcohol has been completely removed. Thus any attempt to recirculate the raffinate phase back to the fermentor must be preceded by an alcohol stripping operation, followed by refortification with malt sprouts.

D. Distillation

No experimental work was performed on the distillation operation since it would involve repeating work already available in the literature. The distillation step was definitely proven feasible by Weiser (121). It consists of steam stripping of the iso-amyl alcohol - water azeotrope from the extract phase plus the usual recovery steps

involving azeotropic distillation to purify the alcohol prior to re-use in the extractor.

DISCUSSION OF THE EXPERIMENTAL RESULTS AS RELATED TO THE PROPOSED NEW PROCESS CALLED FOR IN FIGURE 2

The quantitative results of the experimental program indicate that it is feasible to develop a new process for the production of lactic acid. However, in order to do so it is necessary to discuss the results as they are related to the process, or better still to each specific phase of the process as called for in Figure 2 and pages 33 and 35.

A. Fermentation

In general, the results of the fermentation experiments demonstrate that it is possible to perform the fermentation in such a way as to obtain the lactic acid in the acid form rather than as a salt.

A number of specific factors should be considered in order to perform it at the optimum condition.

The experiments showed an optimum malt sprout concentration of 3-4 percent with respect to fermentation rates and final acid lactate concentrations. This optimum concentration of malt sprouts coincides with the value reported by Peterson (87) for maximum fermentation rates. However, no work has been previously reported relating the nutrient concentration to the final acid lactate concentration.

The experiments showed an optimum fermentation temperature of 42°C which is somewhat lower than the generally accepted optimum of 45°C for lactic acid fermentations at constant pH using Lactobacillus delbrueckii. The three degree difference is understandable since the lactate buffered systems are quite different from the carbonate buffered ones.

The substrate concentration did not show any effect upon the fermentation rate. This coincides with the findings of Finn (98) with fermentations performed at constant pH. It was also found that the final acid lactate was not dependent upon the substrate concentration. No work has been previously reported relating the substrate concentration to the final acid lactate. However, the original glucose concentration will depend upon the conditions at which the fermentor is operated.

The lactate ion buffer concentration was shown to have a considerable effect upon the fermentation rate and the acid lactate concentration at any time during the fermentation. This is very clearly demonstrated in Figure 9 which shows the very strong buffer effect that sodium lactate has upon the fermentation pH at any time during the fermentation. It also shows the effect upon the fermentation rate and acid lactate concentration during the course of the fermentation. The concentration of the lactate ion buffer will be the most important factor to be considered in the fermentation. It will control the pH at which the fermentation is performed and thus controls the fermentation rate. It will also establish the acid lactate concentration in the liquor being removed from the fermentor and the rate of removal of the liquor. By controlling the acid lactate concentration, the original substrate concentration will be also controlled so as not to have a large excess of substrate being recycled around the system. This is particularly important in order to prevent sugar loss by hydrolysis during the process.

The conditions at which the fermentor is operated will be dictated by the economics of the operation. The results of the lactate

buffered fermentations reported in this thesis are sufficient to perform such an economic evaluation.

B. Extraction

The extraction experiments performed in actual fermented liquors demonstrate that the extraction phase of the process is a completely feasible operation. iso-Amyl alcohol is a selective solvent for lactic acid. It does not extract the substrate or the buffer lactate. In case an economic evaluation indicates that the fermented liquor should be concentrated prior to the extraction step, the original substrate concentration should be such that there will not be any substrate being recycled around the system. Any concentration operation should be followed by a settling operation in order to remove part of the cellular proteinic mass. However, the experiments demonstrated the possibility of an easier coagulation in the extract phase. If operated in this way, the extraction should be followed by a settling (coagulation) step prior to the alcohol stripping unit.

The malt sprouts should be filtered from the fermented liquor prior to any concentration or extraction step.

C. Recirculation

The experiments performed with respect to the recirculation step demonstrate that it will be possible to reuse the buffer lactate. It will be necessary to remove the iso-amyl alcohol from the raffinate prior to recycling it back to the fermentor. It will also be necessary to refortify it with malt sprouts. However, this refortification will not require fresh sprouts since Peterson (87) showed that they could be reused. The raffinate will be heated to approximately 203°F during the

alcohol stripping operation. Thus, it will not be necessary to sterilize it prior to returning it to the fermentor.

CONCLUSIONS

The following conclusions were drawn from the experimental results obtained while studying the feasibility of developing the basicly new process for the manufacture of lactic acid.

The quantitative results of the experimental program indicate that it is definitely possible to develop the new process as it was originally proposed. The following pertinent facts were established:

1. The fermentation can be performed in such a way as to obtain the lactic acid in the acid form rather than as a salt. This is possible by controlling the pH of the fermentation through the use of the common ion effect that the lactate salts have upon the extent of ionization of lactic acid. Lactic acid concentrations in the acid form ranging from 11 to 18 grams per liter were obtained by varying the fermentation temperature from 37.5°C to 43.5°C at a lactate ion buffer concentration of 17.6 grams per liter.

2. It is possible to extract the lactic acid from the fermented liquor without any prior purification step. iso-Amyl alcohol proved to be a very selective solvent for this purpose. It does not extract the unfermented sugar or buffer lactate salts. A water-clear extract phase is obtainable.

3. The fermentation may be continued in the raffinate phase provided the alcohol is removed and also provided that the raffinate is refortified with malt sprouts. This shows that the alcohol must remove a nutrient factor which must be reincorporated in the medium.

BIBLIOGRAPHY

1. Underkofler, L. A., Hickey, R. J., Industrial Fermentations, Vol. 1 (1954) The Chemical Publishing Co., Inc., New York, New York.

2. Scheele, K., Quoted by M. Bouillon La Grange, 30 Germinal An. XII, Ann. Chim. Phys. 50, 270 (1780).

3. Fisher, C. H., Filachione, E. M., "Lactic Acid - Versatile Intermediate for the Chemical Industry," U. S. Dept. of Agr., Bur. Agr. and Ind. Chem. A.I.C. 178, May, 1948 (Eastern Regional R. Laboratory).

4. Jungleisch and Godchot, Compt. rend. 144, 979 (1907) 145, 72.

5. Richeter, V. von, Organic Chemistry, Vol. 1, 3rd ed.,1934 790 pp., Nordemann Publishing Co., Inc., New York.

6. Bezzi, S., Riccoboni, L., Sullam, C., "The Products of Anhydrification of Lactic Acid as Type of the Transformation of Cyclic Esters into Linear Polyesters," Mem. Accad. Italia, Classe sci., Fis., Mat., 8, 127 (1937) (C. A. 32, 5379).

7. Moore, S., Dimler, R. J., Link, K. P., Ind. Eng., Chem. An. Ed. 13, 160-3 (1941).

8. Engelhardt, von, Annalen, 70, 242 (1849).

9. Bischoff and Walden, Annalen, 26, 263 (1893).

10. Whitmore, F. C., Organic Chemistry, 1937, 1080 pp., Van Nostrand Co., Inc., New York (p. 358).

11. Watson, P. D., Ind. Eng. Chem. 32, 399 (1940).

12. Pelouze, J. von, Annalen, 53, 144 (1845).

13. Wislicenus, J. von, Annalen 133, 257 (1865), 164, 181 (1872).

14. Jungfleisch and Godchot, Compt. rend. 140, 502 (1905).

15. Peckham, G. T., Jr., "The Commercial Manufacture of Lactic Acid," Chem. Eng. News, 22, 440-43 (1944).

16. Carothers, W. H., Dorough, G. L., Natta, J. H. von, J. Am. Chem. Soc., 54, 761 (1932).

17. Borsook, H., Huffman, H. M., Liu, V. P., "The Preparation of Crystalline Lactic Acid," J. Bio. Chem. 102, 449 (1933). (C. A. 28, 1021 (1934).

18. Thorpe, Edward, A Dictionary of Applied Chemistry, Vol. VII, 4th ed., 1946, Longmans, Green and Co., New York.

19. Filachione, E. M., Fisher, C. H., "Lactic Acid Condensation Polymers. Preparation by Batch and Continuous Methods," Ind. Eng. Chem. 36, 223, 1944 (C. A. 38, 1814).

20. Dimler, R. J., Link, K. P., "Characterization of Lactic Acid as the Benzimidazole Derivative," J. Bio. Chem. 143, 557 (1942) (C. A. 36, 4482).

21. Schatzkes, J., B. P. 173, 479, Nov. 1, 1921 (C. A. 16, 1436).

22. Hamburger, T. U.S.P. 1, 459, 395, June 19, 1923 (C. A. 17, 2887).

23. Olson, E. T., U.S.P. 1, 240, 766, Sept. 18 (C. A. 11, 3278).

24. Jenemann, Joseph A. (To Groselli Chemical Co.) U.S.P. 1, 906, 068, April 25, 1933 (C. A. 27, 3485).

25. Wülfing, R. von, Moller, E., B. P. 356, 600, Dec. 10, 1930 (C. A. 26, 5699).

26. Klapproth, W., B. P. 280, 969, Nov. 22, 1926 (C. A. 22, 3417).

27. Landau, M., Aust. P. 72, 745, Nov. 25, 1916 (C. A. 11, 1017).

28. The Grosselli Chemical Co., Fr. P. 767, 145, July 10, 1934 (C. A. 29, 179).

29. Landau, M., B. P. 7, 998, May 19, 1915 (C. A. 10, 2964).

30. Landau, M., G. P. 294, 726, Sept. 24, 1913 (C. A. 11, 2580).

31. The Grosselli Chemical Co., G. P. 627, 251, March 11, 1936 (C. A. 30, 4179).

32. Noerdlinger, H., Caroselli, A., Berg, L., U.S.P. 924, 494, June 8, 1909, "Purification of Lactic Acid," (C. A. 3, 2114).

33. Noerdlinger, Dr. H., "Chem. Fabrik," Flörsheim addition to G. P. 221, 786, Dec. 2, 1906.

34. Noerdlinger, Dr. H., "Chem. Fabrik," Flörsheim, G. P. 221, 786, Dec. 2, 1906 (C. A. 4, 2718).

35. Macallum, A. D. (To E. I. du Pont) U.S.P. 2, 013, 104, Sept. 3 (C. A. 29, 6906).

36. Lawrie, J. W., U.S.P., 1, 594, 843, Aug. 3, 1926 (C. A. 20, 3171).

37. Haag, Ivan L., (To E. I. du Pont) U.S.P. 2, 013, 096, Sept. 3 (C. A. 29, 6906).

38. Smith, L. T., Claborn, H. V., Ind. Eng. Chem., News Ed., 17, 641 (1939).

39. Klapproth, W., French Patent 644, 314, Nov. 21, 1927 (C. A. 23, 1650).

40. Encyclopedia of Chemical Technology, Vol. 8, pp. 167-180, The Interscience Encyclopedia, Inc., New York.

41. Frankland, P. F., Garner, W. E., "Action of Thionyl Chloride on Lactic Acid and on Ethyl Lactate," J. Chem. Soc. 105, 1101 (1914) (C. A. 8, 2361).

42. Freudenberg, K., Huber, O., "Transformation of d-Lactic Acid into L-alanine," Ber. 58B, 148 (1925) (C. A. 19, 1407).

43. Levene, P. A., Haller, H. L., "Configurational Relationship Between B-hydroxy Butyric Acid and Lactic Acid," Science 62, 356 (1925) (C. A. 20, 579).

44. Purdie, T., Walker, J. W., "Preparation of Active Lactic Acids and the Rotation of their Metabolic Salts in Solutions," J. Chem. Soc. 67, 616 (1895).

45. Szarvas, P., "Observation on the Configuration of α-Hydroxy Acids," Physik, Chem. 188A, 235 (1941)(C. A. 36, 1538).

46. Wood, C. E., Such, J. E., Scarf, F., "Rotary Dispersion of the Esters of Lactic Acis," J. Chem. Soc., 1926, 1928 (C. A. 20, 3445).

47. Clough, G. W., J. Chem. Soc. 1926, 1674 (C. A. 20, 3279).

48. Bean, C. M., Kenyon, J., Phillips, H., J. Chem. Soc. 1936, 303 (C. A. 30, 3784).

49. Woodward, C. F., Chemurgic Digest 9, No. 6, 9 (1950).

50. Smith, L. T., Claborn, H. V., Ind. Eng. Chem. 32, 692 (1940).

51. Rehberg, C. E., Faucette, W. A., Fisher, C. H., "Preparation of Methyl Acetoxypropionate. Reaction of Lactic Acid with Methyl Lactate," Ind. Eng. Chem. 36, 469 (1944) (C. A. 38, 32515).

52. Rehberg, C. E., "Allyl Lactate, Organic Syntheses," 26, 4 (1946).

53. Filachione, E. M., Lengel, J. H., Fisher, C. H., "Preparation of Methyl Lactate. Continuous Alcoholosis of Lactic Acid Polymers," Ind. Eng. Chem. 37, 388 (1945) (C. A. 39, 22849).

54. Filachione, E. M., Lengel, J. H., Fisher, C. H., "Pyrolytic Preparation of Alpha-Carbalkoxyalkyl Acrylates and Methacrylates," J. Am. Chem. Soc., 68, 330 (1946).

55. Filachione, E. M., Fisher, C. H., "Purification of Lactic Acid. Production of Methyl Lactate from Aqueous Solutions of Crude Acid," Ind. Eng. Chem. 38, 228 (1946) (C. A. 40, 1965).

56. Filachione, E. M., Fein, M. L., Lengel, J. H., Fisher, C. H., "Preparation of Alpha, -Carbalkoxylkyl Methacrylates by Pyrolysis of the Corresponding Alpha-Acetoxyisobutyrates," J. Am. Chem. Soc. 70, 526 (1948).

57. Fein, M. L., Fisher, C. H., "Esters of Lactyl Lactic Acid," J. Am. Chem. Soc. 68, 2631 (1946).

58. Fisher, C. H., Filachione, E. M., "Properties and Reactions of Lactic Acid, A Review," U. S. Dept. of Agr., Bur. Agr. and Ind. Chem., A. I. C., 279, Oct. 1950.

59. Kinney, C. H., Ind. Eng. Chem. 32, 559 (1940).

60. Kinney, C. H., J. Am. Chem. Soc. 60, 3032 (1938).

61. Smith, L. T., Claborn, H. V., Ind. Eng. Chem. News Ed. 17, 370 (1939).

62. Wells, P. A., Ward, G. E., "Fermentation Processes," Ind. Eng. Chem. 31, 172-7 (1939) (C. A. 33, 2277).

63. Tatum, E. L., Peterson, W. H., "Fermentation Method for Production of Dextro-Lactic Acid," Ind. Eng. Chem. 27, 1493 (1935) (C. A. 30, 1173).

64. May, O. E., Herrick, H. T., (U. S. Dept. of Agr. Cir., No. 216, May, 1932, 30 pp.), "Fermentation as a Factor in Producing Organic Acids for Chemical Ind.," Chem. and Met. Eng. 42, (3) 142, March, 1935 (C. A. 29, 3103).

65. Bate, J. B., "Chemurgic Processes," Petroleum (London) 8, 134, 154 (1945) (C. A. 40, 3849).

66. Garrett, J. F., "Lactic Acid," Ind. Eng. Chem. 22, 1153-4 (1930).

67. Burton, L. V., "L. bugaricus Using Milk Whey," Food Industries 9, 571 (1937).

68. Inskeep, G. C., Taylor, G. G., Breitzke, W. C., Ind. Eng. Chem. 44, 1955 (1952).

69. Anonymous, "Lactic Acid from Whey: Flow Sheet," Chem. and Met. Eng. 42, 606-7 (1935).

70. Ward, G. E., Lockwood, L. B., Tabenkin, B., Wells, P. A., Ind. Eng. Chem. 30, 1233 (1938).

71. Blastein, Raul J., "Lactic Acid from Molasses by Fermentation with a Sproulated Anaerobium," Rev. asoc. bioquim. argentina 14, 188-90 (1947).

72. Prescott, S. C., Dunn, C. G., Industrial Microbiology, 1940, McGraw-Hill Book Co., New York ("Lactic Acid" pp. 260, 303, 394) (C. A. 34, 6407).

73. Whittier, E. O., Rogers, L. A., Ind. Eng. Chem. 532, May, 1931, 28.

74. Porter, J. R., Bacterial Chemistry and Physiology, 1946, 1073 pp., John Wiley, New York.

75. Whittier, E. O., "Lactose and Its Utilization, A Review," J. Dairy Science 27, 505 (1944).

76. Myers, R. P., Weisberg, S. M., Lactic Acid by Fermentation, 1946, Canadian Patent 432, 878, Jan. 29.

77. Needle, H. C., Fermentation Process for Producing Lactic Acid and Lactates, U. S. Patent 2, 143, 360, Jan. 10, 1939.

78. Eiseman, W., "Production and Place of Lactic Acid," Food Ind. 2, 262-63, 1930.

79. Gunslaus, I. C., Niven, C. F., Jr., "The Effect of pH on the Lactic Acid Fermentation," J. Bio. Chem. 145, 131 (1942).

80. Lee, Sylvan B., Ind. Eng. Chem. 42, 1675 (1950).

81. Hickey, F. C., "The Ionization of Lactic Acid in Aqueous Sodium Chloride Solutions from 0°C to 37.5°C," J. Am. Chem. Soc. 62, 2916-19 (1940).

82. Dietz, A. A., Degering, E. F., Sehopmeyer, H. H., Ind. Eng. Chem. 39, 82 (1947) (C. A. 41, 1208).

83. Fred, E. G., Peterson, W. H., U. S. Patent 1, 485, 844 (1924).

84. Fred, E. B., Peterson, W. H., Ind. Eng. Chem. 13, 211 (1921).

85. Fries, K. W., U. S. P. 2, 474, 046 (1949).

86. Leonard, R. H., Peterson, W. H., Johnson, M. J., Ind. Eng. Chem. 40, 57 (1948).

87. Pan. S. C., Peterson, W. H., Johnson, M. J., Ind. Eng. Chem. 32, 709 (1940).

88. Walter, F. G., The Manufacture of Compressed Yeast, 2nd ed., 316 pp., Chapman and Hall Ltd., London. pp. 135.

89. Liggett, R. W., Koffler, H., "Corn Steep Liquor in Microbiology," Bact. Rev. 12, 297 (1948).

90. Anderson, A. A., Greaves, J. E., Ind. Eng. Chem. 34, 1522 (1942).

91. Liggett, R. W., "The Use of Corn Steeping Liquor in Microbiological Research," J. Bacte. 51, 597 (1946).

92. MacLeod, R. A., Snell, E. E., "Some Mineral Requirements of the Lactic Acid Bacteria," J. Bio. Chem. 170, 351 (1947).

93. Owen, W. L., "Facts about Sugar," 32, 16 (1937) (C. A. 31, 7190).

94. Stevens, K. R., Western, A. E., "Potatoes as a Source of Lactic Acid Fermentation," Proc. Utah Acad. Sci. 19/21, 33 (1941-1943).

95. Laszloffy, A. von, U. S. P. 1, 147, 768 (1915).

96. Bernhauer, K. Rouch, J., Miksch, J. N. Biochem. Z., 320, 178 (1950).

97. Kayser, E., Ann. Inst. Pasteur 8, 736 (1894).

98. Finn, R. K., Halvorson, H. O., Piert, E. L., "Lactic Acid Fermentation Rate. Effect of Continuously Controlled pH," Ind. Eng. Chem. 42, 1857 (1950).

99. Longsworth, L. G., and MacInnes, D. A., "Bacterial Growth with Automatic pH Control," J. Bact. 29, 595 (1935).

100. Longsworth, L. G., and MacInnes, D. A., "Bacterial Growth at Constant pH," J. Bact. 31, 287 (1936).

101. Longsworth, L. G., and MacInnes, D. A., "Bacterial Growth at Constant pH," J. Bact. 32, 567 (1936).

102. Kempe, L. L., Halvorson, H. O., Piret, E. L., "Effect of Continuously Controlled pH on Lactic Acid Fermentation," Ind. Eng. Chem. 42, 1852 (1950).

103. Kempe, L. L., Gillies, R. A., West, R. E., Univ. of Michigan, Ann Arbor, Michigan, Engineering College, Industry Program Report IP-143, December, 1955.

104. Gillies, R. A., Ph.D. Thesis, University of Michigan, Ann Arbor, Michigan, 1955.

105. Lange's Handbook of Chemistry, 1944, 5th ed., p. 853, Handbook Publishers, Sandusky, Ohio.

106. Hillringhaus, F., and Heilman, German Patent 171, 183 (1905).

107. Schapmeyer, H. H., Arnold, C. R. (To American Maize Products Co.) U. S. Patent 2, 350, 370 (1944) (C. A. 38, 49617).

108. Wenker, H. (To Apex Chemical Co.) U. S. Patent 2, 334, 524 (1944) (C. A. 38, 2666).

109. Troupe, R. A., Kobe, K. A., "Kinetics of Methanol-Lactic Acid Reaction," Ind. Eng. Chem. 42, 801-810, 1403-1409 (1950) (C. A. 44, 9351) (C. A. 44, 110656).

110. Byk, A., German Patent 278, 487 (1912).

111. Troupe, R. A., Kobe, K. A., "Kinetics of Methanol Lactic Acid Reaction," Ind. Eng. Chem. 42, 1403-1409 (1950) (C. A. 44, 10656).

112. Horsley, L. H., Anal. Chem. 19, 508 (1947), 21, 831 (1949).

113. Troupe, R. A., Kobe, K. A., "Analysis of Lactic Acid Lactate Esters Systems," Anal. Chem. 22, 545-49, April, 1950.

114. Needle, H. C., Aries, R. S., Sugar 44, No. 12, 32 (1947).

115. Montgomery, R., Ronca, R. A., "Production of Lactic Acid by the Degradation of Carbohydrates," Ind. Eng. Chem. 45, 1136 (1953).

116. Seidell, Atherton, Solubilities of Organic Compounds, Vol. II, pp. 194-5, Van Nostrand, New York, 1941.

117. Ratchford, W. P., Harris, E. H., Fisher, C. H., Willits, C. O., "Extraction of Lactic Acid from Water Solution by Amino-Solvent Mixtures," Ind. Eng. Chem. 43, 778 (1951).

118. Loder, Donald J. (To E. I. du Pont) U. S. P. 2,265, 945 (C. A. 36, 1954).

119. Spengler, O., Goebe, F., German Patent 678, 428, July, 1939 (C. A. 33, 7821).

120. Tindall, John B., (To Commercial Solvents) U. S. P. 2, 223, 797 (C. A. 35, 1806).

121. Weiser, R. B., Extraction, Purification and Processing of Crude Lactic Acid Solutions, Ph.D. Thesis, Ohio State University, 1954.

122. Friedel, C. von, Machuca, F., "Alkaline Hydrolysis of α-Hologenated Propionic Acid," Annalen 120, 285 (1861).

123. Fittig, R., Thomson, G., "Alkaline Hydrolysis of α-Hologenated Propionic Acid," Annalen 200, 79 (1879).

124. Fisher, E., Zemplen, G., "Alkaline Hydrolysis of α-Hologenated Propionic Acid," Ber. 42, 4880, 4891 (1909) 43, 2191 (1910).

125. Senter, G., "Alkalyne Hydrolysis of α-Hologenated Propionic Acids," J. C. S. 95, 1827 (1909), 97, 346 (1910), 99, 1049 (1911).

126. Wislicenus, J. von, Urech, F., "Acid Hydrolysis of Lacto-Nitrile," Annalen, 164, 258 (1872).

127. Wislicenus, J. von, "Acid Hydrolysis of Acetaldehyde Cyanhydrin (Lactonitrile)," Annalen, 128, 11 (1863).

128. Extension of Acid Hydrolysis of Lactonitrile into the Preparation of Lactic Esters, B. P. 257907, 341961, 319043, U.S.P. 1790262.

129. Simpson and Gautier, "Acid Hydrolysis of Lactronitrile," Compt. rend. 65, 416 (1867).

130. Tofel and Friedericks, "Reduction of Pyruvic Acid," Ber. 37, 3187 (1904).

131. Debus, H. von, "Reduction of Pyruvic Acid, "Annalen 127, 333.

132. Wislicenus, J. von, "Reduction of Pyruvic Acid," Annalen 126, 227, 235 (1853).

133. Wurtz, A. von, "Catalytic Oxidation of 1, 2-Propylene Glycol," Annalen 105, 206 (1858), 107, 192.

134. Nef, J. U., "Catalytic Oxidation of 1, 2-Propylene Glycol," Annalen 335, 313 (1904).

135. Evans, W. L., Chem. Review, 31, 537 (1942).

136. Montgomery, R., "The Chemical Preparation of Lactic Acid from Sugars," Scientific Report Series No. 11, Sugar Research Foundation, Inc., New York, January, 1949.

137. Dittmer, K. H., Gustavson, R. G., Ind. Eng. Chem. Anal. Ed. 12, 297 (1940 (C. A. 34, 4237).

138. Bhatnager, S. S., Kapur, A. N., Bhatnager, M. S., J. Indian Chem. Soc. 17, 361 (1940).

139. Oil, Paint and Drug Reporter, Oct. 29, 1956.

140. Martin, A. W., and Tartar, H. V., "The Ionization Constant of Lactic Acid, 0-50°," J. Am. Chem. Soc. 59, 2672-5 (1937).

141. Rogers, L. A., Whittier, E. O., "Limiting Factors in Lactic Acid Fermentation," J. Bact. 16, 211 (1928).

142. Friedman, T. E., Graeser, J. B., "The Determination of Lactic Acid," J. Bio. Chem. 100, 291 (1933).

143. Shaffer, P. A., Somogyi, M., J. Bio. Chem. 100, 695 (1933).

144. Hickinbatham, A. R., "Acidimetric Titration of Lactic Acid Solutions," Analyst 73, 509 (1948).

145. Nims, L. F., Smith, P. K., "The Ionization of Lactic Acid from 0-50°C (From Electromotive Force Measurements," J. Bio. Chem. 113, 145-52.

146. Somogyi, M., J. Bio. Chem. 195, 19 (1952).

147. Neish, A. C., "Analytical Methods for Bacterial Fermentations," National Research Council of Canada, Report No. 46-8-3, Prairie Regional Laboratory, Saskatoon.

148. Freeman, G. G., Morrison, R. T., "Determination of Some Products of Sugar and Molasses Fermentations," Analyst 71, 511.

149. Friedman, T. E., J. Bact. 35, 527 (1933).

150. Hester, A. S., Ladish, J. H., Ind. Eng. Chem. 46, 1738 (1954).

151. MacLeod, R. A., Snell, E. E., "The Effect of Related Ions on the Potassium Requirement of Lactic Acid Bacteria," J. Bio. Chem. 176, 39-52 (1948).

152. MacLeod, R. A., Snell, E. E., "The Relation of Ion Antagonism to the Inorganic Nutrition of Lactic Acid Bacteria," J. of Bacteriology 59, 783-792 (1950).