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Glycerol fermentation of starch

Paul Holland Figard
Iowa State College

NOTE TO USERS

GLYCEROL FERMENTATION OF STARCH

by

Paul H. Figard

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Approved:

Signature was redacted for privacy.
In Charge of Major Work

Signature was redacted for privacy.
Head of Major Department

Signature was redacted for privacy.
Dean of Graduate College

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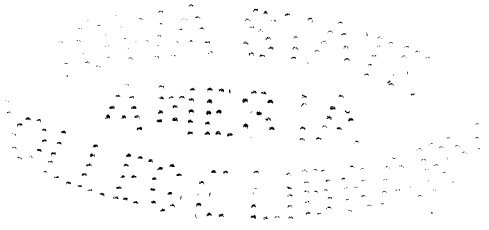

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I. INTRODUCTION

Glycerol, or glycerine as it is commonly called, has become a very important industrial chemical. In addition to the uses for which it is essential there are a wide variety of applications which would lead to expanded consumption of the commodity if it were available in larger quantities at a reasonable cost. At present it is used most extensively in textiles, resins, and explosives. The last use was brought to our attention by the household fat salvage project during the recent war. This conserving of fats also emphasized the fact that our principal source of glycerol was the fat-splitting process. A new synthetic plant which went into operation in 1948 is expected to assure a more adequate supply of pure glycerol.

The fermentation processes for producing glycerol have been restricted mostly to war-time use when the necessity for having it for explosive manufacture overcame considerations of cost. Germany made considerable glycerol by fermentation during the World War of 1914-1918. Their process was developed by Connstein and Lüdecke and consisted of the fermentation of beet sugar in the presence of sodium sulfite as a fixing agent for acetaldehyde. By a rather inefficient method Germany used the process in

24 factories with the smaller ones shipping the fermented slop to the larger ones for the recovery of the glycerol. According to the description given by Lawrie (1928) the output amounted to approximately 1000 tons each month after the factories got into operation on a large scale. A little glycerol was made by fermentation in the United States after the German process had been developed, but here an alkaline method using sodium carbonate was worked out by Eoff. England made glycerol by a fermentation process of Cocking and Lilly which was reported to give very good yields approaching the theoretical value. This method was similar to that of Connstein and Lüdecke but used sodium bisulfite in addition to the sodium sulfite in order to obtain increased yields of glycerol.

In recent years no very drastic changes have been developed in the processes for producing glycerol by fermentation. Most of the later patents in the field are based on only slight modifications of the processes mentioned above. Much work has been done on the problem of recovering the glycerol from fermentation residues. There have been a number of extraction methods worked out which are claimed to be more efficient than the older distillation methods.

Experience has shown that much of the difficulty and expense involved in the fermentation processes arises from

the recovery of the glycerol. The most troublesome factor is the high concentrations of soluble salts present in the fermented beers. For this reason Hickey (1941) investigated the use of less soluble sulfites. He employed magnesium or calcium sulfite instead of the sodium salt. Unfortunately the yields were not as high; however, the recovery of the glycerol should be simpler since the calcium and magnesium salts can be removed by filtration. Sodium ions cannot be gotten rid of so easily, for no common sodium salts are insoluble.

Most of the fermentation procedures for glycerol that are described in the literature use sugar as the raw material, but it would be desirable from the standpoint of initial cost to use starch. There are very few references to the use of starch for this fermentation although it is suggested in a few instances. Connstein and Lüdecke mention the use of a saccharified starch mash in one of their patents, but they give no data on its use.

The theoretical yield of glycerol is approximately 51 per cent, and there are some claims of very nearly this value. However, the majority of the yields reported for large-scale fermentations of commercial size are in the region between 20 and 30 per cent. If the yields are given for glycerol recovered, they are even lower. There is correlation between the size of the inocula and the yields obtained.

The purpose of the investigation on which this thesis is based was to determine the best conditions for a glycerol fermentation process using starch as the fermentation substrate. It was hoped that a process could be developed which might have practical application for commercial glycerol production. To this end most of the work was done with the use of those sulfites which would not increase the concentration of soluble salts enough to make the glycerol recovery too difficult.

II. HISTORICAL

The history of glycerol from its preparation by Scheele in 1779 until 1928 is very well covered in the monograph by Lawrie (1928). For that reason it will be necessary to deal here with only those phases of the subject directly concerned with the present investigation. The principal points of interest will be the fermentation methods for the formation of glycerol.

The literature mentions various synthetic methods for preparing glycerol. Wurtz (1857) made it by reacting 1,2,3,-tribromopropane with silver acetate and hydrolyzing the product, triacetin, with alkali. When it was discovered that propylene could be chlorinated to allyl chloride, it was realized that this reaction could be used as an important starting step for the synthesis of glycerol. The industrial prospects of this method were discussed by Levey (1938), and he concluded that it was economically sound. Williams (1941) also evaluated the economic factors and presented the process as a desirable method for glycerol production. The commercial synthesis using propylene as the starting material was begun finally in 1948 and is described in "Synthetic Glycerine" and "Glycerine by Synthesis", two anonymous articles in Chemical Engineering for October 1948.

A process for producing glycerol by the hydrogenolysis of carbohydrates has been patented by the Association of American Soap and Glycerine Producers, Incorporated (1939). It consists of treating a polyatomic aliphatic alcohol such as sucrose with hydrogen under pressures of about 145 atmospheres and at temperatures above 145° C. Copper aluminate is used as a catalyst, and anhydrous methanol is used for a carrier. The products obtained were 45.8 per cent propylene glycol, 21.5 per cent glycerol, and 6.8 per cent of less volatile glycerol-like compounds.

Pasteur (1858) first reported discovering glycerol as a fermentation product. He found that the normal amount of glycerol formed in fermentations with pure yeast cultures was about 3 g. from every 100 g. of sugar. His results were based on investigations concerned with the production of wines and beers.

Many studies on the mechanism of glycerol formation by yeast have been made. Neuberg and his associates (1917, 1919) did much of the earliest work. He proposed three forms of sugar dissimilation that were possible for yeast. The principal reaction for a normal alcoholic fermentation is expressed by the Gay-Lussac equation:

$$C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + 2CO_2.$$

If, however, an aldehyde-fixing agent is present, the second form of dissimilation takes place according to the following equation:

$$C_6H_{12}O_6 \longrightarrow C_3H_8O_3 + CH_3CHO + CO_2$$

The third type is similar to the second but includes a conversion of the acetaldehyde to ethanol and acetic acid as brought about by alkaline conditions. To fix the acetaldehyde and cause the fermentation to take the second form, Neuberg used such agents as dimedon or the sulfites of sodium, calcium, magnesium, or zinc. Kobel and Tychowski (1928) reported using carbaminic hydrazide (semicarbazide) and thiocarbaminic hydrazide for the same purpose.

The scheme of Embden, Meyerhof, and Parnas for sugar dissimilation provides the most generally accepted explanation for the formation of glycerol by microorganisms. According to this mechanism glucose is first phosphorylated to a hexosediphosphate which then is broken down into two triosephosphates, dihydroxyacetone phosphate and glyceraldehyde phosphate. The last two compounds are in equilibrium with each other. For the normal alcoholic fermentation the glyceraldehyde phosphate is converted by a series of reactions to pyruvic acid, which is decarboxylated to acetaldehyde. The aldehyde is reduced finally to ethanol. In Neuberg's second dissimilation form, where the acetaldehyde is fixed by some sulfite or other agent, the aldehyde cannot be reduced, but instead dihydroxyacetone phosphate is reduced to α-glycerophosphate. This compound decomposes to give glycerol. Porter (1946)

discusses the above fermentation scheme in much greater detail.

As mentioned earlier, in the normal yeast fermentations traces of glycerol are always found. It is supposed that according to the Embden, Meyerhof, and Parnas scheme this is the result of the reduction of some dihydroxyacetone phosphate in the early stages of the fermentation before much acetaldehyde is formed. Once an adequate supply of the aldehyde has been produced, it replaces the dihydroxyacetone as the hydrogen acceptor.

There are numerous books which give discussions of this fermentation mechanism and of the glycerol fermentation in general. Lawrie (1928) reports on much of the experimental work supporting it. The Neuberg and Meyerhof schemes are discussed by Prescott and Dunn (1940) and Porter (1946). A brief review on the production of glycerol by fermentation is found in the article by May and Herrick (1930). A collection of abstracts of articles and patents on the subject was put out by Whalley (1942).

Müller-Thurgau and Osterwalder (1914) were probably the first to observe that when sulfurous acid was added to a fermenting sugar solution it combined with something in the solution. They supposed that the compound which reacted was acetaldehyde, and it was later proven that they were correct. The acetaldehyde-sulfurous acid complex and its sodium salt had been known for quite some time before this.

Connstein and Lüdecke (1919) studied the glycerol fermentation from the standpoint of its industrial possibilities. They first considered an alkaline process. Lawrie (1928) gives their results from using the following alkaline salts: sodium acetate, secondary sodium phosphate, sodium bicarbonate, and ammonium carbonate.

As is so often true with earlier work, no pH values were reported for the solutions used in the work mentioned above. This means that the glycerol yields cannot be correlated with the alkalinity of the fermentation mashes. In many cases it is difficult to decide how much of the effect of the salts is due to the pH and how much to other factors.

Lawrie (1928) mentions several other reagents that Neuberg had used for the alkaline fermentations. These included sodium carbonate, potassium carbonate, potassium bicarbonate, magnesium oxide, tertiary sodium phosphate, and zinc hydroxide. Contamination was found to cause difficulties frequently in some of the fermentations in alkaline medium since many bacteria will grow quite well under these conditions. This trouble was not encountered when high concentrations of sulfites were used. The sulfite has enough antiseptic action to keep contaminants from interfering. Apparently the toxicity is due to the bisulfite ion present in the solutions. Since secondary infections are important considerations for industrial fermentations,

Connstein and Lüdecke turned to the sulfite process.

Sodium sulfite was used to fix the acetaldehyde. Increasing the amount of the sulfite in the medium increased the yield of glycerol. Lawrie (1928) gives a rather detailed report on the results of this study. The data do not show any maximum yield reached by increasing the sulfite concentration. However, the increase in yield is too small to overcome the losses of recovery from the mashes with the high salt contents. When the amount of sodium sulfite is increased too much, the fermentation is slowed down, and the yeast does not function properly. The numerical results are shown in Table 1.

Table 1

Yields of Glycerol with Various Concentrations of Sodium Sulfite*

Sodium sulfite (parts by weight)	Sugar (parts by weight)	Glycerol yield (per cent on sugar)
40	100	23.1
67	100	24.8
80	100	27.3
100	100	30.1
120	100	33.0
150	100	34.6
200	100	36.7

*Adapted from Lawrie (1928)

Connstein and Lüdecke used 40 parts of the sulfite for 100 parts of sugar in their industrial procedure.

The processes were patented by Connstein and Lüdecke in Germany, Hungary, Austria, and the United States. Patent references are listed in Lawrie (1928). There were five claims in the United States patent of Connstein and Lüdecke (1924). The first was for a process for manufacturing glycerol by adding alkaline sulfites (until alkaline reaction) and yeast to sugar and then fermenting the mixture. They made a second claim for a process involving separation of the yeast after the initial fermentation and repetition of the fermentation by adding the separated yeast and alkaline-reacting substances to more sugar. The third claim was for a process using neutral salts of magnesium in a higher amount than necessary as yeast nutrients. Under the fourth claim they suggested adding new portions of sugar after part of the original sugar had been fermented. The last claim was for a process of producing glycerol by fermenting a solution of fermentable sugar in an alkaline-reacting medium. These claims were from the last of all their series of patents taken out in the various countries mentioned above.

Connstein and Lüdecke stated that neither the kind of sugar nor the variety of yeast affected the fermentation. Gehle (1922) disagreed with the statement in regard to the

effect of different yeast strains. The species of yeast used was generally Saccharomyces cerevisiae.

Connstein and Lüdecke (1921, 1924) used molasses and refined and crude sugars, all successfully. They found that the yeast could be recovered and added to the next fermentation without decreasing the yields of glycerol. Although this procedure was not recommended by some other investigators, Connstein and Lüdecke reported the results given in Table 2 for a series of fermentations they ran.

Table 2

Yields of Glycerol Using Yeast from One Fermentation as Seed for the Next*

Times yeast was used	Glycerol yield (per cent on sugar)
1	18.8
2	21.4
3	22.9
4	22.8
5	22.3
6	20.9
7	19.9
8	21.2

*Adapted from Lawrie (1928)

A typical example of the Connstein and Lüdecke process is afforded by the following description: To a solution of one kg. of sugar in 10 liters of water, nutrient salts of

potassium, phosphorus, magnesium, and nitrogen, 100 g. of fresh yeast, and 400 g. of anhydrous sodium sulfite were added. After the mixture was well stirred, it was held at 30° C. for 48 to 60 hours. The alcohol was distilled off, and the sulfite was removed as calcium sulfite before the glycerol was recovered.

These investigators also tried salts other than sulfites, but they all gave lower yields of glycerol. Table 3 indicates the results they obtained.

Table 3

Yields of Glycerol with Various Salts*

Salt (per cent on sugar)		Glycerol yield (per cent on sugar)
Calcium chloride	40	8.2
Ammonium chloride	30	7.3
Sodium chloride	19	8.0
Sodium sulfate	24	6.7
Sodium sulfate	48	8.0
Sodium nitrate	34	5.5
Ferrous sulfate	60	11.8
Ferrous sulfate	120	13.1
Aluminum sulfate	39	9.4
Aluminum sulfate	44	11.6
Aluminum sulfate	80	16.0

*Adapted from Lawrie (1928)

It is interesting to notice that even the salts which give an acid reaction bring about the production of considerable glycerol.

Cocking and Lilly (1922) developed a process which was a modification of the sulfite process of Connstein and Lüdecke. They reported that they could produce glycerol in almost theoretical amounts. In this English process it was found possible to make use of bisulfite in conjunction with normal sulfites to produce a mixture which was neutral in reaction to litmus. Although bisulfites are relatively strong antiseptics and cannot be used alone in large quantities in the glycerol fermentation, they may be introduced in low concentrations into medium containing sodium sulfite without harming the yeast.

Gehle (1922) confirmed Neuberg's work with regard to the equivalence between the acetaldehyde and the glycerol produced by the fermentation of sugar in the presence of sodium sulfite. He found an alteration in the fermentation products with increasing sulfite concentrations and a difference in strains of yeasts in their degree of resistance to the toxicity of the sulfite. By analyzing for aldehyde, glycerol, alcohol, carbon dioxide, and acetic acid, he could account for 85 to 90 per cent of the sugar. The total amount of glycerol produced was equivalent to the amount of acetaldehyde plus a little more comparable to the quantity produced in a normal alcoholic fermentation and an "oxidation value" calculated from the acid production.

The manufacture of glycerol by the use of sulfur

dioxide gas was patented by Barbet (1928). The gas was added to a molasses mash before inoculation and continuously or intermittently after the fermentation had started. Care must be exercised to avoid using the gas in quantities large enough to poison the yeast. This was another case in which an acid mash was used for the glycerol fermentation instead of the more usual alkaline reaction.

Lüdecke and Lüdecke (1929) patented a method which involved following the distillation of beer from one sulfite fermentation with another fermentation. They used a temperature of 30° to 35° C. and a period of two days before the distillation. Magnesium and nickel sulfates were added to the mash. The yields were about 24 to 27 per cent glycerol based on sugar.

Tomoda (1921 to 1929) made an extensive study of the sulfite fermentation for preparing glycerol. He investigated the acetaldehyde-bisulfite complex and its effect on glycerol yields. By increasing the acidity of the fermenting medium Tomoda (1924) found it was possible to decrease the dissociation of the aldehyde complex and raise the yields. The toxicity of the bisulfite ion as contrasted with the aldehyde-bisulfite complex was demonstrated by Tomoda (1928a). The alcohol and glycerol production were observed to occur in parallel by Tomoda (1928b). He worked out mathematical equations to express the amount

of glycerol produced for given concentrations of sugar and sulfite. The velocity constants were determined by Tomoda (1929b) and found to be different functions of the concentration of yeast for different media. He also studied the formation of 2,3-butylene glycol and acetic acid in this fermentation.

Further improvements in the sulfite fermentation were claimed by Imperial Chemical Industries, Ltd. and Lilly (1930) and Giordani (1932). The latter used high concentrations of bisulfite in his fermentations. He obtained yields of 25 per cent glycerol from mashes containing 20 per cent sodium bisulfite.

Most of the work dealing with glycerol fermentations has been done with yeast, but Takahasi and Asai (1933) published an article on the production of glycerol by various species of Mucor. The molds produced normally 3 to 9 per cent of glycerol based on the sugar consumed. Alcohol production approximately paralleled the glycerol production. When sodium bisulfite was added, the glycerol yield was increased. The optimum concentration of the bisulfite was 6 per cent. With this amount the glycerol yield was 21.5 per cent based on the glucose assimilated.

Yeast may be used repeatedly without loss of activity in sulfite fermentations according to Kurbatova and Shakin (1936). They stated that the culture should be grown in

sulfite-free medium between each sulfite fermentation. They also recommended that the yeast be separated from the sulfite medium as soon as the fermentation is finished. This was a confirmation of the results of Connstein and Lüdecke on the continued use of the yeast. In the process of Connstein and Lüdecke, however, it was not specified that intermediate medium without sulfite was necessary.

Rao (1937) experimented with glycerol fermentations of waste cane molasses. He reported on fermentations with Saccharomyces cerevisiae in the presence of alkaline sulfites, carbonates, and bicarbonates. The yields obtained were from 10 to 15 per cent of glycerol based on the sugar.

The Norddeutsche Hefeindustrie A.-G. (1938) patented a glycerol fermentation process using 3 per cent sodium chloride in addition to the sulfite. The method used sugar, sodium chloride, sodium bicarbonate, ammonium sulfate, magnesium sulfate, and yeast and operated at 37° C. and a pH of 7.2 to 7.5. Haehn (1938, 1940) claimed aeration in the presence of oxidation catalysts, such as iron or manganese salts, produced good yields of glycerol.

Corneé (1941) patented a process using 200 to 230 g. of sodium sulfite for 180 g. of sugar and a pH of 8. The fermentation was conducted at 34° to 35° C. for 5 days, and then the solution was distilled at 110° to 120° C. The residue was evaporated and distilled at 10 mm. pressure

and 170° to 180° C. to give a 33 to 35 per cent yield of glycerol.

Hickey (1941) studied the preparation of glycerol using ammonium, calcium, or magnesium sulfites in a sugar medium. Later Fulmer, Underkofler, and Hickey patented a process for the calcium and magnesium sulfites. The study was extended by Lees (1944) to converted starch media using principally magnesium sulfite. The next year Neuberg and Roberts (1946) took out a patent on a sulfite process, which used a mixture of sodium sulfite and sodium bisulfite and gave a yield of 35.2 g. of glycerol from 95 g. of sucrose. One of the most recent patents on a sulfite process was that of Fulmer, Underkofler, and Hickey (1947) using ammonium sulfite.

Considerable work has been done on the production of glycerol by alkaline fermentation methods since the other products in these methods are ethanol and acetic acid, which are more desirable than the acetaldehyde produced by the sulfite process. Eoff developed the best-known American sodium carbonate fermentation. Due to a report during World War I that glycerol was being produced in Germany by a fermentation method, research on this problem was started in the United States. Eoff (1918) obtained a patent on glycerol manufacture by a yeast fermentation in an alkaline medium. He claimed best results by the use of a temperature of 37°

C. and by acclimatization of the organism, Saccharomyces ellipsoideus, variety Steinberg, to the alkaline fermentation conditions. About 20 per cent of the sugar was converted to glycerol when sodium carbonate was used to maintain the degree of alkalinity just below a value which would inhibit the yeast growth. For maximum yields it was necessary to use amounts of alkali up to the endurance limit of the organism. A sugar concentration of about 17.5 g. per 100 ml. of medium was best. The fermented solution from which the glycerol was to be recovered contained 4.4 parts of solids for every part of glycerol, and this made the recovery process difficult and expensive.

Usually the yields obtained from the alkaline fermentations are lower than those of the sulfite processes. Adams (1919) reported only 3 per cent glycerol from sugar using sodium carbonate. Increased glycerol yields were obtained by Eoff, Lindner, and Beyer (1919) from the addition of alkaline reagents, such as sodium and potassium carbonates, bicarbonates, and hydroxides, to a fermentation medium.

McDermott, in the book of Lawrie (1928), gave his theory of the glycerol fermentation of molasses. A shift from an acid reaction to an alkaline one was considered to cause a shift from the first form of Neuberg's schemes to the third form. His theory was that the different hydrogen-ion concentration changed the action of the yeast enzymes

on the carbohydrate being fermented. He also stated that the reason molasses was a good substrate for the alkaline process was the buffer action of the soluble ash content. He pointed out that the buffering effect helped to maintain a more constant pH by lessening the alkalinity when the alkali was added and preventing a rapid lowering of the hydroxyl-ion concentration by the fermentation afterwards. Experimental data were given to show the buffering action of molasses mash as contrasted with a synthetic mash when soda ash was added at intervals. The pH of the molasses mash was more nearly constant and gave a yield of 18.54 per cent as against 15.24 per cent for the synthetic mash.

McDermott stated that a lowered production of glycerol resulted from using those types of molasses having a lower buffer effect. A poor molasses would be improved by adding buffers or arranging the soda dosage to keep the pH more constant. He thought increasing the concentration of the mash might have the same effect since there was an indication from the literature and from practice that high salt or sugar concentrations alone would increase the glycerol production as compared to less concentrated media. McDermott (1929) patented an alkaline glycerol fermentation process using sodium carbonate.

Neuberg and Kobel (1930) studied the fermentation of non-phosphorylated sugar to produce glycerol and pyruvic

acid. Carothers, Hill, and Van Natta (1933) patented another process for manufacturing glycerol by the use of alkali. One of the most important parts of their patent was their distillation method for recovering the glycerol. After removing the alcohol the slop was distilled by spraying it counter current to a stream of superheated steam in a vacuum. The distillate was further purified by mixing with lime and blowing air through the mixture to destroy phenols.

The Norddeutsche Hefeindustrie, A.-G. (1938) described an alkaline method. Magnesium carbonate was used to neutralize the acid formed during the fermentation. Another process patented by Krug and McDermott (1935) made use of ammonia as the alkaline agent. This had the advantage of making the glycerol recovery simpler since ammonia and its salts can be removed. The pH of the mash was adjusted to about 7.3. Using molasses they obtained yields of 18 per cent glycerol based on the sugar.

Hickey (1941) made further investigations on the possibilities of the alkaline fermentation of dextrose by yeast using ammonium hydroxide as the alkalizing agent. He reported that fermentations were unsuccessful when an appreciable ammonium concentration was used in media in which the pH value was above 7. Vokorny (1913) had studied the effect of ammonia on yeast and had also noticed a toxic action.

Takahasi and Asai (1933) in their investigations with molds reported on the effect of addition of alkali. The use of sodium carbonate increased the glycerol yield. Four per cent was the optimum concentration and gave a yield of 23.5 per cent glycerol based on the sugar consumed.

Hodge (1942) patented a process for the manufacture of glycerol by a fermentation of sugar solutions to which ammonia or an ammonium salt was added in amounts above the nutrient requirements; e.g., an ammonia solution equivalent to one-tenth to one per cent by weight of the mash. For this method he suggested a pH of 6 to 7, but in a later patent Hodge (1945a) stated that the more limited range between 6.4 and 7.0 was preferable. The process described in the United States patent by Hodge (1945b) involved growing the yeast in a low-sugar mash, such as ethanol stillage plus ammonium sulfate, with aeration. This gave a sufficient yeast crop in 12 to 24 hours. At this time molasses was added to get a sugar concentration of 15 to 20 g. per 100 ml. Aeration was discontinued, and the pH was brought to approximately 6.6 by adding ammonium hydroxide. From about the twentieth until the thirty-sixth hour of the fermentation, a slurry of freshly-slaked lime, or some other non-toxic neutralizer, was added at intervals to maintain the pH between 6 and 7. After the sixtieth or seventieth hour the beer contained 2.6 to 3.4 g. of glycerol and 5 to

7 g. of ethanol per 100 ml. provided the pH was controlled properly.

The Aktieselskabet Dansk Gaerings-Industri (1944) patented a glycerol and alcohol fermentation process in which concentrated solutions of raw materials containing sugar were fermented by means of at least one part of yeast for each ten parts of sugar while the solution is maintained weakly alkaline for a part of the time at least. The solids in the raw materials comprised at least 82 per cent of fermentable sugar, and the solution contained more than 200 g. of fermentable sugar for each liter of the liquid at the time the fermentation is brought to a close. The pH was preferably 7 to 8 for most of the time and was brought between 6 and 7 towards the end of the reaction. By fermenting 200 kg. of sugar with 100 kg. of press yeast in 600 liters of water at 32° C., with a continuous addition of sodium hydroxide solution, there were obtained after 48 hours 89.6 liters of alcohol and 24.2 kg. of glycerol.

Neish, Blackwood, and Ledingham (1945) reported the production of glycerol and 2,3-butanediol by Ford's strain of Bacillus subtilis when grown at 30° C. on a glucose solution at a pH of 6.0 to 6.8 under anaerobic conditions. By the use of calcium carbonate to control the pH, glycerol yields of 40 moles for each 100 moles of glucose were obtained under laboratory conditions. Blackwood, Neish,

Brown, and Ledingham (1947) found considerable variation in the yields given by different strains of Bacillus subtilis. A commercial process was patented by Neish, Ledingham, and Blackwood (1947). A sterile 5 per cent solution of sugar together with nutrients was fermented at 37° C., and the products included 29.4 per cent glycerol and 28.1 per cent 2,3-butanediol.

Schade and Färber (1947) obtained a patent on a process for the manufacture of glycerol by the fermentation of carbohydrates with yeast in the presence of magnesium carbonate and with a stream of a neutral gas, such as air, passing through the fermenting solution to strip out the more volatile by-products. These by-products could be recovered by scrubbing the exit gas. Hydrolyzed wheat was mentioned as a substrate, and the conditions used were a temperature of 32° C. and a pH of 7.0 to 7.2, controlled by adding the magnesium carbonate. After 26 hours of fermentation 310 g. of pure, refined glycerol were obtained from 1700 g. of reducing sugars. A similar process was patented by Schade (1947) in which a 100 g. of a pressed yeast containing about 72 per cent of water was added to 10 liters of a hydrolyzate of a starch material containing about 10 per cent of total reducing sugar. During fermentation at the usual temperature air was passed through the mixture which was maintained in the neutral range by continuously

neutralizing the acids formed with the addition of a base. A yield of 22 per cent of glycerol based on the fermented sugar and of about 310 g. of yeast with a 72 per cent water content was obtained.

Grover (1947) patented a process for alcohol and glycerol using sodium hydroxide, ammonium sulfate, and secondary ammonium phosphate with initial aeration to give a good yeast growth. A 56-hour fermentation produced 40.6 per cent of ethanol and 8.12 per cent of glycerol by weight on a sugar basis. It was suggested that the spent mash, after separation of the yeast and ethanol, be slopped back to dilute other fermentations thus increasing the amount of glycerol in the mash, facilitating recovery, and improving yields.

III. EXPERIMENTAL

A. Materials

1. Cornstarch

The cornstarch used in these investigations was Buffalo powdered starch obtained from Corn Products Refining Company, Argo, Illinois. It was stored in a tightly sealed metal drum. The moisture content was found to be 11.7 per cent. According to the official acid-hydrolysis method of the Association of Official Agricultural Chemists (1945) this starch analyzed to give a glucose equivalent of 104.2 per cent based on the dry starch or 92.0 per cent based on the wet starch as it was weighed out for use in this work.

2. Steep water

Steep water was used as a nutrient in some of the fermentations and in some of the media for carrying the cultures. It also was obtained from Corn Products Refining Company. It contained 50.0 per cent total solids or 63 g. solids per 100 ml.

3. Yeasts

A strain of Saccharomyces cerevisiae designated as

number 43 (Fleischmann's catalog number 2.15-52) was used for some of the early experiments. It had been used for alcoholic fermentations in these laboratories for many years. A medium containing 5 per cent glucose and 0.5 per cent steep water was employed to carry the culture.

Most of the work was done with massive inoculations. For this purpose ordinary cakes of Fleischmann's fresh yeast were used. They were obtained for each experiment as fresh as possible from grocery stores and were kept in a refrigerator until used.

Several experiments were carried out with a culture of Zygosaccharomyces acidifaciens, American Type Culture Collection number 8766. It was carried on a medium consisting of 20 g. of glucose, 3 g. of Bacto peptone, 0.1 g. of yeast extract, 3 g. of primary potassium phosphate, 3 g. of ammonium sulfate, 0.25 g. of calcium chloride, and 0.25 g. of magnesium sulfate in one liter. Regular transfers of the cultures were made every few days to keep them active.

4. Bacterium

Some fermentations were conducted with Ford's strain of Bacillus subtilis, American Type Culture Collection number 102. It was carried on a medium containing 5 per cent glucose, one per cent calcium carbonate, 0.5 per cent

yeast extract, 0.05 per cent secondary potassium phosphate, 0.05 per cent primary potassium phosphate, and 0.02 per cent magnesium sulfate heptahydrate. The culture was transferred every two days.

5. Sulfites

Two different lots of magnesium sulfite were used in these investigations. They both bore the label of the City Chemical Corporation, New York. The first, used for some of the early experiments, analyzed 56.5 per cent magnesium sulfite, indicating that it was mostly the tetrahydrate. The other lot, which was used for most of the work, analyzed 48.4 per cent magnesium sulfite, corresponding to the hexahydrate. The calcium and ammonium sulfites used were secured from Eimer and Amend, New York. The calcium salt was a dihydrate, and the ammonium sulfite was the monohydrate. A little calcium sulfite and magnesium sulfite were freshly precipitated for use in one experiment. The calcium salt was prepared from calcium chloride and sodium sulfite, and the magnesium sulfite was made from magnesium sulfate and sodium sulfite.

B. Analytical Methods

1. Determination of alcohol

The alcohol determinations were made by distilling the

media from a Kjeldahl flask and collecting 100 ml. of distillate in a volumetric flask. The distillates were distilled a second time from a flask containing 5 grams of a mixture of three parts of sodium sulfite and one part of sodium bisulfite. The second distillate was placed in a constant-temperature water bath at 25° C. and then the specific gravity was determined with a chainomatic Westphal balance.

2. Determination of sugar

The reducing sugar content of the hydrolyzed starch mashes was determined according to the method of Underkofler, Guymon, Rayman, and Fulmer (1943). The reagents were standardized with a series of concentrations of pure glucose solutions. All of the ordinary analyses for the work done for this thesis were carried out in duplicates. Triplicate samples were used where standardizations were involved. The glucose equivalent of the starch was determined by the acid hydrolysis procedure described by the Association of Official Agricultural Chemists (1945) followed by the reducing sugar analysis.

3. Determination of sulfite

Sulfite was determined by titration with a standard 0.1 normal iodine solution. This solution was prepared by

dissolving iodine with potassium iodide in water and standardizing against arsenious oxide. The latter was recrystallized from reagent-grade material using 20 per cent hydrochloric acid.

4. Determination of acetaldehyde

Acetaldehyde was determined by finding the amount of sulfite bound by it. When sulfite is present with acetaldehyde in a weakly acid solution, there may be considered to be one sulfite radical associated with each aldehyde molecule. This bound sulfite is liberated by making the solution weakly alkaline with sodium bicarbonate. Hence, by an iodimetric titration of the free sulfite in weakly acid solution and a further titration after saturating the solution with sodium bicarbonate, the amount of bound sulfite was obtained as the difference between the total and the free sulfite. Tomoda (1929) described the method. Lawrie (1928) and Gehle (1922) also mention methods for determining acetaldehyde.

5. Determination of glycerol

In most of the experiments, where Saccharomyces cerevisiae was used to carry out the fermentations, the glycerol yield was determined by analyzing for the acetaldehyde fixed during the fermentation. The correlation between

the formation of the aldehyde and glycerol was discussed by Neuberg and Reinfurth (1919), and was mentioned in the historical section of this thesis. This procedure gives values slightly below the true amount of glycerol.

For the experiments with Zygosaccharomyces acidifaciens, the above method is not applicable since the glycerol production involves a different mechanism and the acetaldehyde formed is not equivalent in this case. Here a periodate oxidation of the glycerol was carried out using an excess of periodate and adding iodide followed by a thiosulfate titration of the liberated iodine. The procedure followed was that of Wood and Werkman (1940).

In the experiments with Bacillus subtilis a periodate oxidation was again used. The other principal product of these fermentations is 2,3-butanediol, which is also oxidized by the periodate. In the case of the glycerol oxidation formic acid is formed and can be titrated with standard sodium hydroxide. The details of this method are described by Shupe (1943).

C. An Investigation of Various Aldehyde-fixing Agents To Induce the Glycerol Fermentation of Acid-hydroyzed Starch

There are numerous reagents that react with aldehydes to form more or less stable combinations which would probably prevent the reduction of acetaldehyde to ethanol in

fermentation media. Some of these were tried in this experiment even though they couldn't be expected to have much value for industrial fermentations. Thirty grams of starch was used in each flask and hydrolyzed by autoclaving with 300 ml. of 0.1 normal sulfuric acid at 25 pounds steam pressure for 2 hours. Following this the acid was neutralized, and the reagents shown in Table 4 were added. Inoculation was made with a suspension of yeast cakes, and after 3 days glycerol analyses were made by the periodate-oxidation method following removal of the reagents and reducing sugars. The yields of glycerol are given in Table 4.

Table 4

Effect of Various Aldehyde-fixing Agents on Yields of Glycerol

Reagents added to 300 ml. of mash, g.		Glycerol yield, per cent of glucose equivalent
Hydroxylamine hydrochloride	10	3.6
Phenylhydrazine hydrochloride	6	7.2
Semicarbazide hydrochloride	10	7.4
Sodium sulfite	30	29.5

The results in Table 4 indicate that all of the reagents are effective in increasing the glycerol yield above that found normally in yeast fermentations. The use of

phenylhydrazine and semicarbazide gave fair yields. Sodium sulfite was far better than any of the other reagents, and since sulfites are more economical, there would seem to be no reason to consider the other reagents for an industrial process.

D. The Fermentation of Acid-hydrolyzed Cornstarch by *Saccharomyces cerevisiae* in the Presence of Sulfite

1. Acid-hydrolysis of cornstarch

Since Goering (1941) had worked out the conditions necessary for acid-hydrolysis of cornstarch by sulfuric acid, this information was used in preparing media for the glycerol fermentations. An experiment was carried out to determine the effect on the glycerol yields of using different concentrations of sulfuric acid to saccharify the starch. It was decided to use a period of 2 hours and a steam pressure of 25 pounds per square inch for the cooking.

Thirty-gram quantities of starch were weighed out and placed into 500-ml. Erlenmeyer flasks. Three hundred ml. of sulfuric acid solutions of various concentrations, as given in Table 5, was added to each flask. All fermentation media were prepared and fermented in duplicate. The starch was gelatinized by heating the mixtures in a hot water bath until they thickened. They were shaken frequently

during this period of heating to prevent the starch from sticking to the sides and bottom of the flasks. This preliminary gelatinization is probably not necessary, but it avoided the possibility of lumps forming during the cooking to follow. The flasks were then placed in an autoclave and heated for 2 hours at a steam pressure of 25 pounds per square inch. The hydrolyzates were neutralized by the addition of calcium carbonate.

When the temperature of the contents of the flasks had dropped to 60° C., 1.2 g. of mold bran, 0.9 g. of steep water solids, and 30 g. of magnesium sulfite tetrahydrate were added to each flask. After the temperature was down to 30° C., the media were inoculated with 30 ml. of a 24-hour culture of yeast (number 43) grown in a medium consisting of 5 per cent glucose, 5 per cent magnesium sulfite tetrahydrate, and 0.5 per cent steep water. The flasks were placed in an incubator at 30° C. The first glycerol analysis was made on the third day after the inoculation. For this purpose the total volume of the liquid in each flask was measured, and 15-ml. samples were centrifuged. Five ml. of the centrifugate was used for titration with standardized 0.1 normal iodine solution. A few drops of 6 normal hydrochloric acid and one ml. of one per cent starch solution were added before the titration of free sulfite, and excess sodium bicarbonate was added before

Table 5

Effect of Concentration of Sulfuric Acid Used for Hydrolysis of Starch on Yields of Glycerol and Ethanol

Conc. of acid, normality	Glycerol yield, per cent of glucose			Ethanol yield, per cent of glucose
	3rd day	4th day	5th day	
0.01	10.5	12.3	12.3	16.3
0.02	14.5	17.5	17.4	18.8
0.05	14.6	18.8	19.5	18.6
0.1	14.2	18.9	19.8	20.7
0.2	13.8	18.9	19.9	21.8

the second titration as explained in the section on methods of analysis. These analyses were repeated on the two following days. Analysis for alcohol was made on the fifth day. The yields found are given in Table 5. These yields were calculated on the basis of the glucose equivalent to the 30 g. of starch, as found by the analysis mentioned under the section on materials, plus the 1.5 g. of glucose contained originally in the inoculum.

On the basis of the results of this experiment it was decided to adopt 0.1 normal sulfuric acid as the concentration for hydrolysis in future experiments. When the analyses were made on the third day, it was evident that the fermentations were not complete, for there was an active evolution of carbon dioxide from the flasks. The results of the first set of analyses were rather misleading as to the best concentration of acid. By the fourth day the

maximum yield of glycerol, 18.9 per cent of sugar, was found in the two highest concentrations of acid. On the fifth day there was still little difference between the yields of the two highest concentrations. The fermentations were nearly complete by this time since the gassing had nearly stopped and the analyses of the flasks from the lower acid concentrations showed little change from the fourth day.

The color of the hydrolyzates was darker at the higher acid concentrations. This indicated some destruction of sugar by caramelization. From the glycerol yields of Table 5 there is little choice between the 0.1 and 0.2 normal acid. The latter was discarded because of the evidence of more sugar decomposition even though this wasn't indicated in the yields.

For an industrial process considerably higher pressures and a shorter time would be used for this hydrolysis step. The time could be shortened from hours to a matter of minutes, but the high pressures required were not readily available in the laboratory. Ruf, Stark, Smith, and Allen (1948) described an acid-hydrolysis process which is satisfactory for industrial purposes.

Hayek and Shriner (1944) present a possible process for hydrolyzing starch by sulfurous acid. It would seem that it might be applicable to the sulfite glycerol fermentation. For this reason an experiment was undertaken

to test it.

Six grams of starch was placed in each of three pyrex tubes used for Carius halogen determinations. Enough of a standard solution of sulfur dioxide in water (titrated against a standard iodine solution) was added with additional water to give 60 ml. of acid solutions containing 0.340, 0.687, and 1.044 g. of sulfur dioxide, respectively, in the three tubes. It had been calculated that these concentrations would give final concentrations in the liquid phase of 0.5, 1.0, and 1.5 g. of sulfur dioxide per 100 g. of water after the tubes were sealed and heated to 135° C. Part of the sulfur dioxide would be driven from the liquid phase into the gas phase above which had a volume of 50 ml. The calculation was made from an extrapolation to 135° C. of the vapor pressure data for sulfur dioxide solutions as given in volume III of the International Critical Tables, page 302. The weight of sulfur dioxide which would be in the gaseous phase was determined approximately from the gas law equation using a pressure obtained from summing the extrapolated partial pressures and subtracting the partial pressures of air, water, and sulfur dioxide at the temperature in the tube when it was sealed. This quantity of sulfur dioxide was added to the amount desired in the liquid phase to give the values used above.

Six 50-ml. Erlenmeyer flasks were also prepared in

duplicate with 3 g. of starch and 30 ml. of 0.02, 0.05, or 0.1 normal sulfuric acid in each to compare with the sulfurous acid hydrolysis. The flasks and sealed tubes were placed in an autoclave and heated for 2 hours at a steam pressure of 30 pounds per square inch. After cooling, the tubes were opened, and a little magnesium carbonate was added to all of the tubes and flasks to partially neutralize the acid. Samples were taken for sugar analyses, and then the contents of the tubes were divided between duplicate 50-ml. Erlenmeyer flasks. To all of the flasks enough magnesium sulfite hexahydrate was added to give 3 g. of the sulfite in each flask. They were inoculated with 1.5 ml. of an active yeast suspension and incubated at 30° C. Since large inoculations were used, the fermentations seemed to be complete by the third day. At this time glycerol analyses were made by the method described for sulfite fermentations. Table 6 presents the results of this experiment.

With the sulfuric acid the amount of conversion to sugar increased with the concentration of the acid as in the previous experiment. However, even with the lowest concentration the conversion was quite good. The glycerol yield was less for the lowest concentration even though the yields were calculated on the basis of the sugar found by the analysis.

Table 6

Comparison of Sulfurous and Sulfuric Acid Hydrolysis of Cornstarch for the Glycerol Fermentation

Concentration of hydrolytic agent	Conversion of starch to glucose, per cent	Glycerol yield, per cent of glucose
Sulfuric acid		
0.02 normal	93.9	14.0
0.05 normal	96.1	16.1
0.1 normal	99.0	16.1
Sulfurous acid		
0.5 g. SO_2/100 g. H_2O	67.2	13.5
1.0 g. SO_2/100 g. H_2O	72.5	8.8
1.5 g. SO_2/100 g. H_2O	80.2	7.6

With the sulfurous acid the amount of conversion to sugar was not so good but did increase with the concentration of the acid. Hayek and Shriner (1944) report some better conversions than these; so it is probable that a higher concentration or higher pressure would have given better hydrolysis. The glycerol yields were disappointing. At the higher concentrations of sulfur dioxide the results would indicate that the free sulfur dioxide or bisulfite ion was inhibitng the fermentation.

This sort of inhibition was observed also in some experiments to be described later where sulfur dioxide was used. Probably a more complete neutralization of the sulfurous acid to give a higher pH would have produced better results. According to the patent of Barbet (1928), however,

the medium could still be acid when using sulfur dioxide. Although sulfurous acid could probably be used as the hydrolytic agent for a glycerol fermentation of starch, the above data indicate that the sulfur dioxide process would be more difficult to carry out than the sulfuric acid hydrolysis, and it does not seem as suitable for industrial use.

2. Effect of addition of nutrients to glycerol fermentations

The addition of many of the salts commonly used as nutrients in yeast fermentations was tried by Lees (1944) and found to have little effect on the glycerol fermentation. Various less common salts have been reported by investigators to stimulate yeast fermentations. Some of these were used for this experiment at the concentrations shown to be effective for other fermentations. The procedure was similar to that described in the first experiment on hydrolysis with various concentrations of sulfuric acid. In this case the concentration of sulfuric acid used for hydrolysis of the starch was 0.1 normal. Thirty grams of cornstarch was added to each 500-ml. Erlenmeyer flask with 300 ml. of the acid. The starch was gelatinized in a hot water bath, and the flasks were autoclaved for 2 hours at 25 pounds steam pressure. After the flasks had cooled, the acid was neutralized as before, and 30 g. of magnesium sulfite tetrahydrate and the phosphate, arsenate

or tartrate were added. Thirty ml. of a culture of yeast grown for 24 hours in a sulfite medium was used for the inoculum of each flask. The glycerol yields found as the fermentations progressed are shown in Table 7.

Table 7

Effect of Addition of Salts on Yields of Glycerol

Salt	Glycerol yield, per cent of glucose equivalent				
	3rd day	4th day	5th day	6th day	7th day
None	2.3	2.4	3.1	4.2	6.8
$K_2HPO_4.3H_2O$, 0.5 g./100 ml.	2.3	2.5	4.2	7.0	9.2
Na_2HAsO_4, 0.005 molar	1.9	1.9	2.2	3.2	4.6
$K_2C_4H_4O_6$, 8 mg./100 ml.	2.2	2.3	3.3	4.2	6.9

These data indicated that the glycerol fermentation is slow and the yields are low without the addition of nutrients. The use of steep water and mold bran in the first experiment on sulfuric acid hydrolysis resulted in much better yields than any of these. The addition of phosphate increased the yield, and the arsenate decreased it, while tartrate had no effect.

Up to this time most of the inoculations had been made with liquid cultures of yeast rather than yeast cakes even though it had been reported by Hickey (1941), Lees (1944),

and others that massive inoculations gave faster fermentations and better yields. It was first thought that the use of yeast cakes was not practical industrially, but, since massive inoculations and reuse of yeast are practical in industrial fermentations of sulfite waste liquor and wood hydrolyzates, similar procedures should be applicable for glycerol fermentations. Hence, massive inoculations from yeast cakes were used in subsequent experiments. The cakes were suspended in water to give about one cake in each 45 ml. of suspension, and 15 ml., or one-third of a cake, was added to the usual 300 ml. of fermentation medium. For the present experiment on the effect of some citrates, 30 g. of cornstarch was weighed into each 500-ml. Erlenmeyer flask, and 300 ml. of 0.1 normal sulfuric acid was added. The starch was gelatinized, autoclaved at 25 pounds steam pressure for 2 hours, and cooled. The acid was only partly neutralized with calcium carbonate so that the medium resulting would be slightly acid. Thirty grams of magnesium sulfite and 24 mg. of the citrates were added. Fifteen ml. of the suspension of yeast cake was used for inoculation. The fermentations progressed rapidly at 30° C., and analyses were made on the third day when the rate of evolution of carbon dioxide had slowed down. The results of this experiment are shown in Table 8.

Table 8 indicates that the small amounts of the citrates do not influence the glycerol yields very much.

Table 8

Effect of Addition of Citrates on Yields of Glycerol

Citrate added	Glycerol yield, per cent of glucose equivalent
None	21.1
Magnesium	21.7
Sodium	20.9
Ammonium	21.8

The magnesium and ammonium salts gave slightly better yields than the control, but the most important point about these data is the fact that the fermentations were all rapid with high yields as a result of the massive inoculations with yeast cakes. Complex nutrients might be expected to increase the yields more than salts would. For this reason various nutrients and enzyme preparations were tried to investigate their effect either as nutrients or as saccharifying agents. The procedure was similar to that of the last experiment except that the nutrients and enzymes were added after the hydrolysis and partial neutralization when the medium was still at 60° C. to give the enzymes a chance to exert a further saccharifying action if possible.

The addition of the substances listed in Table 9 increased the ethanol yields some but did not appreciably

Table 9

Effect of Addition of Complex Nutrients and Enzyme Preparations on Yields of Glycerol and Ethanol

Substance added, per cent of starch		Glycerol yield, per cent of glucose equivalent	Ethanol yield, per cent of glucose equivalent
Nothing		24.7	15.9
Yeast extract	1.5	25.2	16.2
Malt	4.0	24.9	17.9
RHozyme S	0.2	25.0	18.1
Amylase concentrate	4.0	25.4	18.5
Mold bran	4.0	25.0	18.4
Bran	4.0	24.5	18.5
Corn steep liquor	5.0	24.4	18.5

change the glycerol yields. There was a slight increase of glycerol but not enough to compensate for the cost of the nutrients. For this reason none were used in subsequent work with massive inoculations.

3. Studies on the glycerol fermentation of cornstarch with various sulfites

Except for the first preliminary experiment the work so far had been done with magnesium sulfite exclusively as the fixing-agent. Two other sulfites which could be easily removed after fermentation without increasing the soluble salt content are calcium sulfite, which is not very soluble, and ammonium sulfite, which could be decomposed and eliminated by heating. Thirty- and 60-g. quantities of each of

the three sulfites were compared in this experiment. The procedure was the same as in previous runs. Thirty grams of cornstarch and 300 ml. of 0.1 normal sulfuric acid were placed in 500-ml. Erlenmeyer flasks, and gelatinization and autoclaving at 25 pounds per square inch steam pressure for 2 hours followed. Enough calcium carbonate was added to give a final pH of 6, and each flask was inoculated with 15 ml. of the yeast cake suspension. The results are given in Table 10.

Table 10

Comparison of Magnesium, Calcium, and Ammonium Sulfites as Fixing-Agents for Glycerol Fermentations

Sulfite	Glycerol yield, per cent of glucose equivalent
30 g. Magnesium sulfite	23.4
60 g. Magnesium sulfite	23.1
30 g. Calcium sulfite	4.6
60 g. Calcium sulfite	4.1
30 g. Ammonium sulfite	1.3
60 g. Ammonium sulfite	1.6

From the data of Table 10 it was evident that magnesium sulfite gave much greater yields than either the calcium or ammonium salt. The fermentations with ammonium sulfite appeared very sluggish with little evidence of carbon dioxide

evolution. Since the use of 60 g. of the sulfites did not give increased yields over those obtained with 30 g. except with the ammonium salt, 30 g. may be considered a sufficient amount.

Neuberg and Reinfurth (1919) thought that the use of freshly precipitated calcium sulfite in fermentations resulted in higher acetaldehyde fixation than did the use of a commercial anhydrous salt. It was decided to try both freshly precipitated calcium sulfite and freshly precipitated magnesium sulfite in comparison with the commercial products. Thirty grams of starch and 300 ml. of 0.1 normal sulfuric acid were placed in 500-ml. Erlenmeyer flasks. The starch was gelatinized, autoclaved for 2 hours at 25 pounds steam pressure, and cooled. The acid was partly neutralized to give a final pH slightly above 6, and the sulfites were added. The freshly precipitated sulfites were prepared from sodium sulfite and calcium chloride or magnesium sulfate. Inoculation was made with 15 ml. of the usual yeast cake suspension. The data for these fermentations are given in Table 11. The yields are those determined on the third day.

The results in Table 11 indicate that freshly precipitated calcium sulfite may be some better than the commercial product, but there was no improvement in yields using the freshly precipitated magnesium sulfite. Actually it seemed

Table 11

Comparison of Freshly Precipitated and Commercial Sulfites as Fixing-Agents for Glycerol Fermentations

Sulfite	Glycerol yield, per cent of glucose equivalent
Magnesium sulfite hexahydrate	
30 g. commercial	22.5
30 g. freshly precipitated	21.8
60 g. freshly precipitated	22.0
Calcium sulfite dihydrate	
30 g. commercial	4.1
30 g. freshly precipitated	4.8
60 g. freshly precipitated	4.8

that the physical state of the sulfites, in regard to their moisture content and how finely powdered they were, determined how much they tended to cake and form lumps which in turn probably affected the yields. This would be expected especially in these fermentations, where there was no constant stirring but only occasional shaking of the flasks.

For a commercial process both the sulfite and yeast would probably be recovered and used over in subsequent fermentations. This was tried, starting with 30 g. of starch and 300 ml. of 0.1 normal sulfuric acid in 500-ml. Erlenmeyer flasks. The starch was gelatinized and autoclaved for 2 hours at 25 pounds steam pressure. After the

acid had been partially neutralized to give a final pH of 6.3, the magnesium sulfite was added, and inoculation with 15 ml. of the yeast cake suspension followed. The fermentations were incubated for 65 hours at 30° C. before analyses were made. Then the media were filtered, and the cakes of sulfite and yeast were added to another set of flasks of hydrolyzed starch medium prepared as before. To one pair of duplicate flasks no further additions of sulfite or yeast were made. To the others various amounts of sulfite or yeast or both were added as shown in Table 12. These second fermentations were again incubated for 65 hours at 30° C. and analyzed to give the results in Table 12.

Table 12

Glycerol Yields of Successive Fermentations Using Sulfite and Yeast Recoverd from First Fermentation in the Second Fermentation

First fermentation		Second fermentation		
Sulfite added g.	Glycerol yield, per cent of glucose equiv.	Sulfite added g.	Yeast added, cakes	Glycerol yield, per cent of glucose equiv.
60	20.8	0	0	15.1
60	20.7	0	1/3	20.1
30	20.2	30	0	17.1
30	19.9	30	1/3	19.7
30	20.4	15	0	16.8
30	20.0	15	1/6	19.0

The second fermentations gave reasonably good yields, indicating that the sulfite and yeast can be recovered and used over. The addition of more yeast resulted in better fermentations than where no more was added. There were two possible reasons for this. For one the filtration process used to recover the sulfite and yeast from the first series was very slow so that the yeast was often dry and probably not very active by the time it was added to the second series, and for another the conditions of these rapid fermentations with massive inoculations very likely give little growth of the yeast.

When calcium sulfite was used as the fixing-agent, much poorer glycerol yields were obtained than with magnesium sulfite. It was desired to find the effect of adding magnesium ions to a calcium sulfite fermentation. Thirty grams of starch was weighed into 500-ml. Erlenmeyer flasks, and 300 ml. of 0.1 normal sulfuric acid was added. The medium was autoclaved for 2 hours at 25 pounds steam pressure. The acid was neutralized with calcium carbonate, and then 30 g. of calcium sulfite dihydrate and various amounts of magnesium sulfate were added to the flasks. Inoculation was made as usual, and glycerol was determined after 65 hours of incubation. The data are reported in Table 13.

The results in Table 13 indicated that the magnesium

Table 13

Effect on Glycerol Yields of the Addition of Magnesium Sulfate to Calcium Sulfite Fermentations

Magnesium sulfate heptahydrate added, g.	Glycerol yield, per cent of glucose equiv.
0	5.0
6	6.3
12	7.5
18	8.0
24	8.8
30	9.5

sulfate increased the glycerol yields from calcium sulfite fermentations. The yields increased up to the highest concentration of magnesium sulfate used. This is interesting from a theoretical standpoint, but for a practical industrial process it is not significant. The highest yield is still less than half of that obtained with magnesium sulfite.

Since the last experiment showed that magnesium ion improved calcium sulfite fermentations, it was decided to try various mixtures of the sulfites as fixing-agents. The starch was hydrolyzed in the usual manner. After the acid had been partly neutralized with calcium carbonate, the sulfites were added in the amounts shown in Table 14. Glycerol analyses were made on successive days with the results collected in Table 14.

The results indicated that the mixtures do not give as good yields as the magnesium sulfite alone. The yields

Table 14

Effect of Various Mixtures of Magnesium and Calcium or Ammonium Sulfites on Yields of Glycerol

Magnesium sulfite $6\ H_2O$, g.	Calcium sulfite $2H_2O$, g.	Ammonium sulfite H_2O, g.	Glycerol yield, per cent of glucose equivalent		
			First day	Second day	Third day
30	0	0	12.0	19.1	20.5
60	0	0	11.5	17.6	21.2
20	10	0	11.6	18.7	19.0
15	15	0	11.6	15.8	15.9
40	0	20	6.7	6.2	6.2
15	0	15	3.5	3.1	3.2

from the magnesium and calcium sulfite mixtures were fairly good but decreased as the proportion of the calcium salt was increased. The calcium sulfite was probably contributing very little to the aldehyde fixation. Results from other experiments would lead to this conclusion. Ammonium sulfite appears actually to inhibit the fermentation when added with magnesium sulfite. The glycerol analyses gave lower values on the second and third days than were obtained on the first day. These flasks exhibited practically no gas evolution or other signs of fermentative activity. Except for the fermentations containing ammonium sulfite the yields increased from day to day, but the process seemed to be about complete on the third day. This was judged from the rate of evolution of carbon dioxide which had slowed

down by the third day. A few check analyses made on the fourth day confirmed this observation. Some of the flasks gave lower values for the glycerol content by the fourth day.

It is known from many reported yields in the literature that sodium sulfite will give better yields than these obtained from magnesium sulfite. If the yields with magnesium sulfite could be increased by adding a little sodium sulfite without adding enough to increase the soluble sodium salt content very much, it might be practical to use such a mixture. This was investigated in the following experiment. The hydrolyzed starch was prepared as usual. After the media had cooled, the calcium carbonate was added to partially neutralize the acid. Addition of the sulfites followed. On the third day glycerol analyses were made. The results are presented in Table 15.

Table 15

Effect of Various Mixtures of Magnesium and Sodium Sulfites on Yields of Glycerol from Acid Media

Magnesium sulfite hexahydrate, g.	Sodium sulfite heptahydrate, g.	Glycerol yield, per cent of glucose equivalent
30	0	21.3
24	8	22.6
20	13	23.2
15	20	20.5
10	26	16.6
0	39	5.1

Although the yields did increase with increasing sodium sulfite for the two smallest additions, there was a decrease with larger additions. The explanation for this decrease is most likely the fact that the acid used for hydrolysis was not completely neutralized leaving an acid medium to which the sulfites were added, and although this is favorable for fermentations with magnesium sulfite alone, it gave enough bisulfite ion with the more soluble sodium sulfite to be toxic to the yeast.

Since the last experiment did not tell what it was desired to learn from it, another one was set up in which it was made certain that the media were alkaline. The starch was hydrolyzed in the usual manner. The acid was completely neutralized. The sulfites were added in the quantities shown in Table 16. All of the media had pH values above 7. When the fermentations were analyzed after 65 hours, the yields of glycerol were found to be as shown in Table 16.

Table 16

Effect of Various Mixtures of Magnesium and Sodium Sulfites on Yields of Glycerol from Alkaline Media

Magnesium sulfite hexahydrate, g.	Sodium sulfite heptahydrate, g.	Glycerol yield, per cent of glucose equiv.
30	0	19.8
20	10	23.7
15	15	25.8
0	30	28.5

With this set of fermentations there was a steady increase in the yield of glycerol as the proportion of the sodium sulfite was increased. The rise, however, was too gradual to make it advisable to add sodium sulfite to magnesium sulfite fermentations. The increase in yield would not compensate for the greater difficulty of recovery from the beer with a higher soluble salt content.

Before the study of the use of mixtures of sulfites was given up, an experiment was made using ternary mixtures of ammonium, calcium, and magnesium sulfites. The usual 10 per cent cornstarch mashes were prepared. Acid hydrolysis was carried out, and the rest of the preparation for fermentation was as usual. The sulfites were added in the amounts shown in Table 17. On the third day after inoculation samples were taken, and their glycerol content was determined. The results are presented in Table 17.

Table 17

Effect of Various Mixtures of Ammonium, Calcium, and Magnesium Sulfites on Yields of Glycerol

Magnesium sulfite hexahydrate g.	Calcium sulfite dihydrate, g.	Ammonium sulfite monohydrate, g.	Glycerol yield, per cent of glucose equiv.
20	20	20	6.7
30	15	15	2.9
30	20	10	2.8
40	15	5	6.0
60	0	0	22.0

The data of Table 17 indicated that in all cases of the mixtures, the fermentations were greatly inhibited. No further work was done on mixtures of sulfites as fixing-agents. Attention was turned now to the effect of pH of the media on the fermentations with the different sulfites.

4. Effect of pH on the glycerol yields obtained from acid-hydrolyzed starch with various sulfites

As a result of previous experiments, a slightly acid medium had been found desirable for the fermentations with magnesium sulfite. According to the opinion of Hickey (1941) the fermentations with ammonium sulfite should also be on the acidic side of neutral, for he thought that molecular ammonia or ammonium hydroxide in solution when the media had a pH value above 7 was toxic for the yeast. The object of this investigation was to see how the glycerol yields changed with the pH of the medium in the presence of the magnesium, calcium, or ammonium sulfites.

In the first of this series of experiments magnesium sulfite was employed. The mashes were prepared in the usual manner. The only changes in the procedure came after the hydrolysis of the starch. The sulfite was added, and then the pH was adjusted. For measuring the pH a glass electrode pH-meter was used. Concentrated solutions of hydrochloric acid or sodium hydroxide were added in small quantities to give the desired pH. The flasks were then

Table 18

Effect of pH on the Yields of Glycerol and Ethanol from the Fermentation of Acid-hydrolyzed Starch in the Presence of Magnesium Sulfite

pH			Glycerol yield per cent of glucose equiv.	Ethanol yield per cent of glucose equiv.
Initial	At 20 hrs.	At 40 hrs.		
5.0	5.2	5.0	13.9	0.3
5.5	5.5	5.5	9.8	0.9
6.0	6.1	6.0	22.5	18.5
6.5	6.4	6.5	22.7	16.5
7.0	6.8	6.9	19.1	22.2
7.5	7.0	7.1	17.5	20.5

inoculated in the usual way and placed in the incubator. At 20-hour intervals the pH was measured and readjusted to the desired value where necessary. The data are collected in Table 18.

The above data illustrate the fact that the glycerol yields are influenced quite markedly by the pH of the medium used. This is undoubtedly due to the concentration of bisulfite ion produced from the sulfite at the different pH values. The optimum pH seemed to be between 6.0 and 6.5 with a rather rapid decline in yields when the pH fell below 6.0. Practically no alcohol was formed in the media at pH 5.0 and 5.5. There were still fair yields of glycerol at these low pH values but much below the best yields. The amount of glycerol formed at pH 5.0 was definitely greater

than that at 5.5, probably because of the change in the rate of reaction of the yeast enzymes with the pH.

A similar series of fermentations was carried out with both calcium and ammonium sulfites. The same pH range was examined for the calcium sulfite, but for the ammonium salt a more acid range was used because of the observation of Hickey that alkaline media were unsatisfactory for use with ammonium sulfite. The data obtained are summarized in Table 19, giving averages for the duplicate fermentations.

Table 19

Effect of pH on the Yield of Glycerol from the Fermentation of Acid-hydrolyzed Starch in the Presence of Calcium or Ammonium Sulfites

pH			Glycerol yield, per cent of glucose equivalent
Initial	At 20 hrs.	At 40 hrs.	
Calcium sulfite dihydrate, 30 g.			
5.0	4.9	5.0	4.5
5.5	5.7	5.5	6.0
6.0	5.7	5.8	4.3
6.5	6.1	6.1	3.7
7.0	6.2	6.3	3.2
7.5	6.4	6.5	2.8
Ammonium sulfite monohydrate, 60 g.			
4.5	4.2	4.4	1.4
5.0	4.5	4.6	1.5
5.5	5.4	5.4	1.3
6.0	5.5	5.6	1.1
6.5	6.3	6.4	1.6
7.0	6.7	6.9	0.9

For the calcium sulfite fermentations there is again a regular variation of the yields of glycerol with the pH of the medium used. It is once more evident that magnesium sulfite was much superior to either the calcium or ammonium salt in bringing about good yields of glycerol. The difference between the effectiveness of the magnesium and calcium sulfites is probably due to the difference in the concentration of the sulfite ion in solution which results from their solubilities. With the less soluble calcium sulfite the optimum pH was at a more acid reaction of about 5.5. The yield at this point was better than those reported earlier in this thesis for calcium sulfite fermentations; so the conditions of pH used in previous experiments had probably not been optimum. About all that can be said about the data for ammonium sulfite is that the fermentations were very poor. The lowest yield was at the highest pH.

5. Effect on glycerol yields of the addition of sulfur dioxide to magnesium sulfite fermentations

Since it might be desirable for an industrial process to use sulfur dioxide to control the acidity of glycerol fermentations, the effect of adding sulfur dioxide was investigated. Actually the pH of the media does not change much during the fermentation if the initial adjustment was made to a value near the optimum, so that little sulfur dioxide would be needed for this purpose. In the following

experiments more sulfur dioxide was used than would be needed for simply controlling pH.

For the first experiment flasks of hydrolyzed starch were prepared as usual. Thirty grams of magnesium sulfite hexahydrate was added to each. Sulfur dioxide was bubbled through the media in half of the set of flasks for a short time. The pH was adjusted to the values shown in Table 20, and inoculation was made with one-third of a yeast cake for each flask. At 20-hour intervals the sulfur dioxide treatment was repeated, and the pH of all the flasks was readjusted. The results of the glycerol analyses made on the third day are given in Table 20.

Table 20

Effect of Intermittent Addition of Sulfur Dioxide on Yields of Glycerol from Acid-hydrolyzed Starch in the Presence of Magnesium Sulfite

pH	Sulfur dioxide	Glycerol yield, per cent of glucose equiv.
6.0	-	23.1
6.0	+	17.6
6.5	-	22.8
6.5	+	22.1
7.0	-	20.2
7.0	+	20.0

The data from Table 20 showed that the yields were less for those fermentations to which the sulfur dioxide was

added. Also it was evident that as the pH was increased the amount of inhibition decreased. The concentration of bisulfite ion is probably the significant factor in the inhibition observed. On the basis of these data it would appear that sulfur dioxide could not be used in very high concentrations in any glycerol fermentation medium with an acid pH.

Another experiment was carried out in which a continuous slow addition of sulfur dioxide was used. The gas was bubbled very slowly through the usual magnesium sulfite medium in a pair of duplicate flasks. The gas coming from these two flasks was bubbled through the contents of another pair containing the same medium initially. This second pair of flasks had more gas passing through them since there was considerable carbon dioxide evolved from the first pair. The magnesium sulfite was stirred up some by these gas bubbles, and hence the question of the stirring effect was brought up. To check this another pair of flasks was stirred with motor-driven stirrers to keep the magnesium sulfite suspended in them. The results of this series of fermentations are presented in Table 21.

The results in the last table indicate that stirring is advantageous for the fermentation. The flasks which were not stirred were shaken nevertheless several times a day as in all of the previous experiments. Since there

Table 21

Effect of Continuous Addition of Sulfur Dioxide and Stirring on Yields of Glycerol

Treatment	Glycerol yield, per cent of glucose equivalent
(1) None	22.1
(2) Sulfur dioxide bubbled through	17.0
(3) Exhaust from (2) bubbled through	22.4
(4) Stirred	23.2

was no apparatus available in the laboratory to stir a large number of flasks uniformly, however, in subsequent experiments stirring was not resorted to unless specifically mentioned. The sulfur dioxide again inhibited the fermentations into which it was first introduced. The apparently increased yield observed in the second pair of flasks may be due to acetaldehyde carried over by the gas from the first flasks. Although all this work with sulfur dioxide was not of a quantitative nature, the results indicated that further refined investigations of the addition of sulfur dioxide to magnesium sulfite fermentations were not warranted. In this connection it might be well to recall the inhibition observed in experiments reported in an earlier section of this thesis where sulfur dioxide was used to hydrolyze the starch for fermentations.

6. Effect of varying the mash concentration and temperature on the glycerol yields obtained from acid-hydrolyzed starch mashes

For commercial purposes it is desirable to use as high a mash concentration as will give good yields of glycerol. The variation of the yields with starch concentration was next investigated. A series ranging from 5 to 20 per cent was set up.

The various required quantities of starch were weighed into 500-ml. Erlenmeyer flasks, and 300 ml. of 0.1 normal acid was added. The starch was hydrolyzed, and the acid was partially neutralized. After taking samples for a sugar analysis, the fermentations were carried out in the usual manner. The magnesium sulfite was used in varying amounts equal to the weight of the starch in order to keep the ratio of sulfite to substrate constant. The results are collected in Table 22.

Table 22

Effect of Varying the Mash Concentration on the Yields of Glycerol

Concentration of mash, per cent	Conversion of starch to glucose, per cent	Glycerol yield, per cent of glucose	
		Second day	Third day
5	100.2	23.6	23.4
10	95.7	22.6	22.5
15	94.6	18.8	19.2
20	93.6	16.5	17.1

From the above data it is obvious that the yields drop off with increasing starch concentrations. The starch conversion to sugar also gets poorer at the higher concentrations of mash. The fermentations seemed to be quite complete on the second day. At the two lower starch concentrations there was a slight decrease of yields by the third day. It was to be expected that the more concentrated mashes would take longer to ferment even though the inoculum was used in amounts proportional to the weight of starch in the flasks.

A further investigation was made testing the influence of temperature of incubation on the glycerol yields from a series with different starch concentrations. Two series of starch mashes were made up and run as usual except that one was incubated at 30° C. and the other at 37° C. The results are given in Table 23.

Table 23

Effect of Temperature on Glycerol Yields of Various Concentrations of Starch Mashes Hydrolyzed with Acid

Temperature, degrees Centigrade	Concentration of mash, per cent	Glycerol yield, per cent of glucose equivalent
30	5	22.0
30	10	21.5
30	20	17.0
37	5	22.5
37	10	21.9
37	20	17.2

The results presented above indicate that 37° C. was more favorable for the glycerol fermentation than 30° C. The yields were a little better at the higher temperature with all concentrations of starch. Although this is above the optimum temperature for yeast growth, the enzymes involved in the fermentation undoubtedly function better at this temperature. However, the lower temperature was still used in the following experiments reported in this thesis. The decreasing glycerol yields with increasing concentrations of starch were again evident in this experiment. A 10 per cent mash was used in most of the subsequent work.

7. Effect on glycerol yield of delaying the addition of sulfite to the fermentation

It was felt that in these fermentations with massive inoculations there was very little growth of the yeast. The fermentations appeared to be carried out largely by the enzymes contained in the yeast introduced as the inoculum. It would seem desirable to have the yeast growing actively at the time the fermentation was begun. One way to accomplish this would be to inoculate the hydrolyzed starch medium and delay the addition of the sulfite until the yeast had a chance to become active. The fermentations were prepared in the usual way except that the addition of the magnesium sulfite was delayed for various periods of time after inoculation as shown in Table 24. Sixty-five hours after the

Table 24

Effect of Delayed Addition of Magnesium Sulfite on Yields of Glycerol

Time between inoculation and addition of sulfite, hours	Glycerol yield, per cent of glucose equivalent
0	23.5
0.5	22.0
1	21.3
2	20.0
20	4.0
24	2.8

inoculation the glycerol was determined in the usual manner. The data are collected in Table 24.

From the data presented in Table 24, it is apparent that delaying the addition of sulfite caused only considerable lowering of the glycerol yields. This result was expected for the cases where the sulfite addition was delayed for the longer times but not for the short time intervals. Even a delay of 30 minutes resulted in a lower glycerol yield. Still smaller intervals of time might have showed an increase, but it did not seem that much activation of the yeast could be obtained by this method. Apparently from the data the fermentation of the substrate to ethanol starts off so rapidly that any activation of the yeast is more than offset by the loss of substrate to ethanol production. It is interesting to note that the ethanol fermentation must be

nearly complete at the end of one day, for, when the sulfite was added at that time, the glycerol yield was about that normally found in the alcoholic fermentation. Even with the 20-hour addition the glycerol found was only a little above normal.

8. Effect on glycerol yields of acclimatization of yeast to magnesium sulfite

For the early work in this thesis before massive inoculations were used, the yeast cultures had been acclimatized to the sulfite by transferring the yeast from the usual sugar medium to one containing magnesium sulfite before the culture was used for inoculating the fermentations. In the experiments using yeast cakes they had only been suspended in water and added directly to the media to be fermented. It was thought advisable to see if the acclimatization to sulfite would improve the fermentations with yeast cakes. The mashes were made up in the usual way. Inoculations were made with 15 ml. of suspensions of one-fourth of a yeast cake in a medium consisting of 5 per cent glucose, 5 per cent magnesium sulfite hexahydrate, and 0.5 per cent steep water. The suspensions had been incubated at 30° C. for various lengths of time as shown in Table 25.

The results in Table 25 show that acclimatization of the yeast cake suspensions to sulfite has no appreciable

Table 25

Effect on Glycerol Yields of Acclimatization to Sulfite of Yeast Cake Inoculum

Length of time of acclimatization, hours	Glycerol yield, per cent of glucose equivalent
0	23.3
2	23.0
4	23.1
6	23.2
9	22.9
24	23.0

influence on the glycerol yields. The yields were calculated on the glucose equivalent to the starch weighed out plus the small amount of glucose which was used in the inoculum suspensions. With these massive inoculations acclimatization is not useful as it is with the liquid cultures.

9. Effect on glycerol yields of activating the yeast before inoculation

Neither delaying the addition of the sulfite nor acclimatization to sulfite improved the action of the yeast in bringing about the glycerol fermentation. Another attack on the problem was to try to activate the yeast a little before it was used to inoculate the fermentations. The usual hydrolyzed starch medium with magnesium sulfite was prepared, and inoculation was made with one-fourth of a

yeast cake which had been suspended in a medium consisting of 5 per cent glucose and 0.5 per cent corn steep liquor and incubated at 30° C. for various periods of time as shown in Table 26. The results of glycerol analyses made 65 hours after inoculation are collected in Table 26.

Table 26

Effect of Activating Yeast for Various Times on Yields of Glycerol

Time of activation period, hours	Glycerol yield, per cent of glucose equivalent
0	20.8
2	21.0
4	22.7
6	23.4
9	23.1
24	21.2

From the above data it appears that better glycerol yields can be obtained by activating the yeast before inoculation. A maximum yield of 23.4 per cent was gotten when a 6-hour activation period was used. This length of time was probably about that required for the large amount of yeast to exhaust the small amount of sugar available. Even after 24 hours the yeast gave an increased yield although the sugar must have been used up for quite some time.

E. The Fermentation of Acid-hydrolyzed Cornstarch by *Bacillus subtilis*

1. Effect of mash concentration and nutrients on the yields of glycerol obtained from acid-hydrolyzed starch by *Bacillus subtilis* fermentations

Bacillus subtilis has been shown to produce glycerol and 2,3-butanediol from sugar solutions according to the work of Blackwood, Neish, Brown, and Ledingham (1947). It should be possible to adapt this fermentation to acid-hydrolyzed starch mashes. The fermentations were prepared in a similar manner to those for yeast fermentations except that various concentrations of starch were tried, and salts and nutrients in the following concentrations were used: 0.05 per cent secondary potassium phosphate, 0.05 per cent primary potassium phosphate, 0.02 per cent magnesium sulfate heptahydrate, one per cent calcium carbonate, and 0.5 per cent yeast extract or one per cent corn steep liquor. Inoculation of each 250-ml. Erlenmeyer flask containing 150 ml. of medium was with 10 ml. of a 24-hour culture of *Bacillus subtilis* grown at 37° C. The results of glycerol analyses, made on the fifth day by periodate oxidation and titration of formic acid produced, are collected in Table 27.

The results from Table 27 indicate that the yields fall off as the starch concentration is increased. Corn steep liquor as a nutrient source gave slightly better yields than

Table 27

Effect of Mash Concentration and Nutrients on Yields of Glycerol from Bacillus subtilis Fermentations

Mash concentration, per cent	Nutrient	Glycerol yield, per cent of glucose equivalent
5	Yeast extract	12.0
7.5	Yeast extract	10.2
10	Yeast extract	9.6
5	Steep liquor	13.3
7.5	Steep liquor	10.6
10	Steep liquor	10.4

yeast extract. This would be desirable since the steep liquor would be a more economical nutrient for industrial use. These yields obtained in this experiment were much lower than those reported by Blackwood, Neish, Brown, and Ledingham (1947). The fermentations appeared slow, and it is possible that if they had been allowed to continue for a longer time higher yields would have been found.

2. Effect on glycerol yields of adding sulfite to Bacillus subtilis fermentations

In view of the fact that the 2,3-butanediol produced by the Bacillus subtilis fermentation has little demand, it was thought desirable to investigate the effect of sulfite in fixing the acetaldehyde before the glycol was formed if the organism could tolerate it. Fifteen grams of cornstarch was weighed into 250-ml. Erlenmeyer flasks and hydrolyzed

with 150 ml. of acid in the usual manner. The acid was neutralized, and one per cent of corn steep liquor was added. The media were sterilized and cooled, and sterile calcium carbonate, salt solution, and magnesium sulfite as shown in Table 28 were added. After fermenting for 6 days the mashes were analyzed for glycerol and acetaldehyde. The data are collected in Table 28.

Table 28

Effect of Various Amounts of Magnesium Sulfite on Yields of Glycerol from Bacillus subtilis Fermentations

Magnesium sulfite hexahydrate, g.	Glycerol yield, % of glucose equivalent	
	Total	Equivalent to acetaldehyde
0	10.5	0.9
4	8.2	1.7
8	5.8	2.0
12	5.0	2.2
16	2.8	2.4
20	3.2	2.8

The data in Table 28 indicate that the total amount of glycerol dropped off more or less regularly as the sulfite concentration was increased. At the same time the amount of acetaldehyde increased with increasing concentration of sulfite. With the two highest amounts of sulfite the total glycerol content was only slightly greater than the glycerol equivalent to the acetaldehyde. The effect of the sulfite

was certainly one of inhibition of the normal fermentative activity of the Bacillus subtilis.

3. Effect of pH and stirring on the glycerol yields from Bacillus subtilis fermentations of hydrolyzed starch

Since the pH was found to exert a great deal of influence on the yields of glycerol obtained from yeast fermentations, it was decided to test this point in regard to the Bacillus subtilis fermentations of hydrolyzed starch. A 7.5 per cent starch mash was hydrolyzed with 0.1 normal sulfuric acid in 250-ml. Erlenmeyer flasks. The acid was neutralized, and one per cent corn steep liquor was added. The pH was adjusted to the values shown in Table 29. In some cases one per cent calcium carbonate was added to control the pH near 6. After sterilization and the addition of nutrient salt solution the flasks were inoculated with 10 ml. of a 24-hour culture of Bacillus subtilis grown at 37° C. Some of the flasks were stirred. Glycerol analyses were made after the fermentations had been going for a week in a 37° C. incubator. These data are in Table 29.

A pH of 6 was more favorable than the higher values. The use of calcium carbonate to hold the pH near 6 and stirring, especially where the carbonate was used, improved the yields. These fermentations were more vigorous than the former ones probably in part due to the higher temperature of incubation.

Table 29

Effect of pH and Stirring on Yields of Glycerol from *Bacillus subtilis* Fermentations of Hydrolyzed Starch

pH	Stirring	Glycerol yield, per cent of glucose equivalent
7.0	-	13.5
6.5	-	16.2
6.0	-	18.6
Calcium carbonate	-	20.7
6.0	+	18.8
Calcium carbonate	+	22.0

F. The Fermentation of Acid-hydrolyzed Cornstarch by *Zygosaccharomyces acidifaciens*

1. Effect of nutrients and sulfite on *Zygosaccharomyces* fermentations

In the experiments reported by Nickerson and Carroll (1945) with *Zygosaccharomyces* fermentations considerable glycerol was obtained from sugar without the use of any aldehyde-fixing agent. The following series of fermentations was made to see how hydrolyzed starch would serve as a substrate and to see what the effect would be of adding sulfite and using other nutrients to replace the peptone and yeast extract mixture used by Nickerson and Carroll. Two hundred ml. of 10 per cent starch medium was used in 250-ml. Erlenmeyer flasks. After hydrolysis the acid was neutralized and salts were added to give the following

concentrations per liter: 3g. of primary potassium phosphate, 3 g. of ammonium sulfate, 0.25 g. of calcium chloride, and 0.25 g. of magnesium sulfate. The other nutrients and magnesium sulfite were added as indicated in Table 30. The mashes were then inoculated with 15 ml. of a 2-day culture of Zygosaccharomyces acidifaciens and allowed to ferment for 10 days in a 30° C. incubator. The results of glycerol and acetaldehyde analyses are collected in Table 30.

Table 30

Effect of Sulfite and Various Nutrients on Yields of Glycerol and Acetaldehyde from Zygosaccharomyces Fermentations

Magnesium sulfite hexahydrate g.	Nutrient g.	Glycerol yield, per cent of glucose equivalent	
		Total	Equiv. to acetaldehyde
0	Peptone, 0.6 + yeast extract, 0.02	7.7	0.6
0	Yeast extract, 0.5	8.0	0.4
0	Corn steep liquor, 1	8.3	0.4
20	Peptone, 0.6 + yeast extract, 0.02	8.4	8.0
20	Yeast extract, 0.5	8.7	8.4
20	Corn steep liquor, 1	8.6	8.6

The results of Table 30 indicated that sulfite increased the production of glycerol slightly. Corn steep liquor seemed to be a satisfactory nutrient. According to the work

of Nickerson and Carroll (1945) the metabolism of this yeast follows to a considerable extent Neuberg's third form of fermentation. The addition of sulfite to fix the acetaldehyde results in eliminating this form, for practically all of the glycerol found in these cases was equivalent to the acetaldehyde.

2. Effect on glycerol yield of pH and the use of massive inoculations in Zygosaccharomyces fermentations

Massive inoculations and pH were known to affect the glycerol yield with Saccharomyces. It was thought that the effect with Zygosaccharomyces should be studied. The usual starch media was prepared in 200-ml. quantities. After hydrolysis and neutralization of the acid, one ml. of corn steep liquor and a nutrient salt solution were added to each flask to give the concentrations mentioned in the last experimental series. Magnesium sulfite was added to half of the series of flasks, and the pH was adjusted to the values indicated in Table 31. A considerable quantity of the yeast had been grown in a molasses-salts medium to provide for massive inoculations. It was activated for 6 hours and used in amounts corresponding to those used for the ordinary yeast cake inoculations where large inocula are indicated in Table 31. For the small inoculations the ordinary liquid cultures were used as in the last series. Table 31 shows the results of glycerol and acetaldehyde analyses as made on the fifth and tenth days.

Table 31

Effect of pH and Massive Inoculations on Yields of Glycerol from Zygosaccharomyces Fermentations

Magnesium sulfite hexahydrate g.	Inocula	pH	Glycerol yield, per cent of glucose equivalent			
			Fifth day		Tenth day	
			Total	Aldehyde-equivalent	Total	Aldehyde equiv.
0	Large	7.0	8.5	0.7	8.3	0.4
0	Large	6.3	7.8	1.4	7.7	0.6
0	Small	7.0	4.3	0.9	8.0	0.9
20	Large	7.0	21.1	20.3	21.0	19.9
20	Large	6.3	22.8	21.9	22.5	21.0
20	Small	6.3	7.6	6.8	14.7	13.6

The use of massive inoculations with sulfite gave yields on the fifth day which were comparable to those obtained with the Saccharomyces fermentations. The higher pH was more favorable without sulfite and the lower with sulfite. Again, it is evident that although this yeast will produce about 8 per cent glycerol without sulfite, the glycerol produced in the presence of sulfite is only that equivalent to the acetaldehyde fixed by the sulfite.

IV. DISCUSSION

The yields of glycerol obtained from either of the yeast cultures in the presence of magnesium sulfite and from the Bacillus subtilis fermentations of acid-hydrolyzed starch were nearly the same. The bacterial fermentation was slower than those with the yeast if massive inoculations of yeast were used. About a week was needed using Bacillus subtilis, whereas the yeast fermentations were usually complete by the third day.

For raw materials the starch, acid, and culture were required in each process. With the bacterial fermentation a nutrient, such as corn steep liquor, and calcium carbonate were also needed. With Saccharomyces magnesium sulfite was required, and with Zygosaccharomyces it was also desirable since it increased the glycerol yield by two and a half times.

From the standpoint of the salt content of the beer the glycerol recovery should be simplest from the fermentations with the Bacillus. This is because no sulfite was used. With Zygosaccharomyces the sulfite could be omitted, but the increased yield of glycerol obtained with its use would probably more than compensate for the increased cost of recovery. The difference in by-products in the processes might be expected to affect the ease of recovery of glycerol also.

Each of the three organisms could yield different by-products. With the Saccharomyces fermentation the principal by-products were ethanol and acetaldehyde. With the bacterium they were 2,3-butanediol and lactic acid. The by-products from the Zygosaccharomyces fermentation depended upon whether sulfite was used or not. In the presence of sulfite the principal secondary products were again ethanol and acetaldehyde, but in the absence of sulfite they were ethanol and acetic acid.

The best yields from the yeast fermentations in the presence of sulfite would correspond to about 25 lbs. of glycerol, 20 lbs. of ethanol, and 10 lbs. of acetaldehyde from 100 lbs. of starch. At present prices the three products would be worth $3.81, $1.01, and $1.15, respectively, or a total of $5.97 while the starch would cost $4.67 and the magnesium sulfite needed would cost about $5.00 but would be largely recoverable for reuse. With the Bacillus subtilis fermentation one could expect to get 25 lbs. of glycerol, 25 lbs. of 2,3-butanediol, and 10 lbs. of lactic acid from 100 lbs. of starch. The glycerol and lactic acid would have a total value of $5.66, but there is no market for the 2,3-butanediol at present although it does have a potential value. The starch would again cost $4.67, and there would be an additional expense of about $0.36 for calcium carbonate and nutrients. From

Zygosaccharomyces fermentations of 100 lbs. of starch without sulfite the yields would be 10 lbs. of glycerol, 10 lbs. of ethanol, and 5 lbs. of acetic acid, according to the figures of Nickerson and Carroll (1945) with only 57 per cent utilization of the substrate. These products would be worth $1.52, $0.50, and $0.38, respectively, or a total of $2.40, and the starch cost of $4.67 would indicate that better utilization of the substrate would be necessary to make this fermentation of any commerical interest. In the presence of sulfite the Zygosaccharomyces gives a fermentation which corresponds almost exactly to that of the Saccharomyces. Since this investigation was centered around the production of glycerol, no study was made of the by-products in the absence of sulfite.

V. SUMMARY AND CONCLUSIONS

1. Acid-hydrolyzed starch has been found to provide a suitable substrate for glycerol fermentations. In all cases tried the starch medium gave practically as good yields as those reported for sugar medium. The use of acid-hydrolyzed starch as the substrate for glycerol fermentations would reduce initial cost.

2. Glycerol can be produced from acid-hydrolyzed starch mashes by the use of various common aldehyde-fixing agents other than sulfite. The glycerol yields are lower than those obtained from sulfite fermentations. From the consideration of economics and recovery sulfite is better suited for an industrial process.

3. The addition of nutrients to glycerol fermentations of acid-hydrolyzed starch is unnecessary if large inocula are used. The fermentations are brought about apparently by the enzymes associated with the inocula, and there is very little proliferation of the yeast. Since the nutrients are useful only as a supply of growth factors for the yeast, they are not needed for the fermentations.

4. Magnesium sulfite proved to be much more satisfactory than either calcium or ammonium sulfite for the production of glycerol by the fermentation of acid-hydrolyzed

starch. The addition of magnesium ion to a calcium sulfite fermentation increased the glycerol yield somewhat but did not give nearly as good results as the use of magnesium sulfite. Magnesium sulfite and yeast can be used over for successive fermentations if care is taken to maintain the activity of the yeast.

5. The use of mixtures of magnesium, calcium, and ammonium sulfites gives poorer yields of glycerol than the use of magnesium sulfite alone. Ammonium sulfite actually inhibits the fermentation so that its use in mixtures prevents the other sulfites from giving their normal yields of glycerol. Addition of sodium sulfite to a magnesium sulfite fermentation increases the yield, but the concentration of soluble salts is also increased thereby making the recovery of the glycerol more difficult.

6. The pH of the fermentation media influences the yields of glycerol. For magnesium sulfite the optimum value lies between 6.0 and 6.5, and for the calcium sulfite it is about 5.5. When ammonium sulfite is used, the variation of glycerol yields with pH is not great for pH values between 4.5 and 6.5.

7. The percentage yields of glycerol decrease when the initial concentration of starch is increased. This effect was observed in fermentations conducted at both 30° C. and 37° C. It was found that the yields of glycerol

obtained at 37° C. were higher than those at 30° C. for the same starch concentrations.

8. The degree of activity of the yeast used as inocula affects the yields of glycerol when large inocula are used. The activity of the yeast from commercial yeast cakes can be increased by suspending them in a glucose-corn steep liquor medium and incubating the suspension for several hours before it is used for inoculation of the fermentation mash. The addition of sulfite to the suspension to acclimatize the yeast to the sulfite before inoculation does not improve the glycerol yields.

9. Glycerol can be produced from acid-hydrolyzed starch by Bacillus subtilis, Ford's strain. The yields are about as good as in the yeast fermentations, and no aldehyde-fixing agent is required. When sulfite is added to the fermentations, the yield of glycerol decreases, although the amount of acetaldehyde found as a product increases.

10. The yeast Zygosaccharomyces acidifaciens ferments acid-hydrolyzed starch without an aldehyde-fixing agent to give a considerable amount of glycerol. It has been found that the addition of sulfite to the fermentation increases the glycerol yields and the amount of acetaldehyde. With the use of sulfite an acid reaction is desirable, but without sulfite a neutral pH is better.

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