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A Microbiological Process Report

Acetone-Butanol Fermentation of Starches

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The production of acetone and butanol by the Weizmann fermentation process has previously been described in some detail by Killeffer (1927), Gabriel (1928), Gabriel and Crawford (1930) and Prescott and Dunn (1949). The complete description of the modern industrial process using starch-containing material and its resulting problems has not been previously reported. The following data are presented in an effort to bring up-to-date the literature on this subject.

MICROORGANISMS USED

Clostridium acetobutylicum is the organism commonly used in the industrial acetone-butanol fermentation of starchy mashes. It is a spore-forming rod. Its inactive stage consists of a rod containing a spore, or a free spore by itself. There are several other types of organisms similar to *C. acetobutylicum* which deserve some mention. They are *Clostridium roseum* and *Clostridium felsineum*. These organisms, when grown in a corn or starch mash, produce a pink-colored mash and produce yields of acetone and butanol equivalent to about one-half to three-quarters of that produced by *C. acetobutylicum*. *C. felsineum* is used in the flax retting industry for its enzymatic powers of hydrolyzing the pectin, causing a separation of the fiber from the cortex and wood. Jean (1939) has patented the use of this culture to ferment an admixture of garbage and grains to n-butyl alcohol, acetone and ethyl alcohol.

Numerous strains of *C. acetobutylicum* have been isolated. Some are characterized by different fermentations of carbohydrates, different ratios of solvents, immunity to bacteriophage attacks and visual characteristics of the fermentation. The isolation of new cultures of *C. acetobutylicum* may be accomplished in many ways. One consists of the introduction of a small amount of soil, manure, roots of leguminous plants, cereals, decayed wood, corn stalks, sewage or river-bottom-mud, into a sterile 4 per cent corn mash tube. The corn mash tube is then heated for two minutes at 212 F in a boiling water bath or Arnold steamer to destroy any vegetative forms present; only the spores survive. These tubes are then cooled to 98 F and are allowed to incubate at 98 F in a constant temperature incubator for several days. Usually, in 24 hours' time the tubes begin to produce gas and the corn starch undergoes fermentation. By noticing the head or cap (if one is formed) or by smelling the tube, one experienced in the art can determine with considerable certainty whether an acetone-butanol organism is present. If neither of the above-mentioned criteria is evident, the usual custom is to discard the tube. If, however, the culture has a sweet butylic odor or the corn has formed a head on top, we then proceed to plate out the culture. This involves making dilutions of the unknown culture and adding these dilutions to Petri plates containing sterile malt agar, prepared especially for the isolation of such cultures. These plates are then incubated under strict anaerobic conditions for 48 to 72 hours at 98 F. Upon observation of the plates, numerous colonies are picked from the plate and carried through a series of tests in corn mash, finishing up with a quantitative test on 8 per cent to 10 per cent corn

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mash for solvent production, head formation, slime, pH, riboflavin content, time of fermentation and other characteristics. The best solvent-yielding cultures are allowed to sporulate by letting them incubate for about five days. This sporulated culture then is aseptically added to a sterile sand, soil and calcium carbonate mixture and dried for several days. This comprises the stock culture of the organism and has been found by experience to be the best storage method for industrial use.

Another method of isolation which has worked extremely well is to add 10 gram samples of starch-containing cereals to small Erlenmeyer flasks containing 100 ml of sterile water. The contents are then pasteurized by holding the flasks in a water bath at 135 F for 35 minutes. The flasks are then removed, cooled and incubated at 98 F. Observation of the previously mentioned characteristics are made and the same procedure for isolation and testing of the organism is followed. It should be pointed out that considerable differences are noted between various strains of *C. acetobutylicum* and their ability to produce acetone and butanol. A complete bacteriological description of *C. acetobutylicum* was made by McCoy, Fred, Peterson and Hastings (1926) and by Weyer and Rettger (1927).

Raw Materials Used

The following carbohydrates or carbohydrate-containing raw materials have been used either alone or in admixture in the acetone-butanol fermentation with *C. acetobutylicum:* Maize, kaffir corn, rye, wheat, rice, milo, cassava, horse chestnuts, artichokes, granular flour, potatoes, sweet potatoes, hydrolyzed peanut hulls and oat hulls, hydrolyzed corn cobs, pentoses such as arabinose and xylose, Irish moss, heated whey, grape musts, waste sulfite liquor, jawari, manioc, dari, bajra, hexoses, molasses and sugar syrups. Some of these raw materials can be used simply by rendering the starch soluble; others require hydrolysis. Thus corn or cereal meal may partially be replaced by sucrose, glucose, molasses, hydrol, or hydrolyzed cellulose or pentosans.

Fermentation Process

The modern fermentation is carried out with moderate changes in procedure from that of Weizmann's original patented process. The classic material used is corn (or maize). The corn is first screened; particles of dust and iron are removed by screening and passing the grain over a magnetic separator. Sometimes the grain is treated to remove the germ from which corn oil may be expelled. This corn oil may also be recovered after fermentation, either from the germ by expelling, or from the various fractions of the dried stillage by extraction with oil solvents. The kernel remaining, or the whole corn if the germ is not removed, is ground in either roller mills or hammer mills into a fine powder. If potatoes are used, they are hammered into a pulpy, watery mass and diluted to a consistency which permits pumping. Ground corn is mixed with water and steam to give a concentration of 8 to 10 per cent corn. This mash was formerly cooked in "Batch Cookers", a term given to a 10,000-gallon horizontal pressure vessel equipped with an agitator or rake and numerous steam injection ports. The mash could be cooked up under pressure using agitation and be blown down before cooling. The more modern type of mash cooking is the "continuous" method such as described and patented by Carnarius (1947), or a process similar to that described by Pfeifer and Vojnovich (1952). Using the Carnarius process, the mash is heated with a steam injector and pumped under pressure through a series of from four to six elongated, vertical pressure-detention-tanks of 13,000-gallon capacity, moving downward slowly in each of these along a spiral path. When leaving the last tank the mash is sterile.

The following procedure is usually followed. The entire cooking system is filled with mash and recirculation is accomplished by means of pumps. A temperature of 250 to 260 F is maintained for about 60 minutes, at which time the mash is drawn off and fresh mash fed to the system continuously. The starch in the corn is rendered soluble and the entire mash is sterilized. The cooked mash is pumped from the cookers—at which time it may be diluted by adding water at 180 F—into a series of double-pipe coolers which will lower the temperature of the heated mash to about 98 F. It is necessary to have all the lines, pumps, coolers and every pipe and valve with which the mash or culture comes in contact sterilized by means of steam. The cooled mash is then pumped into the sterile final fermentation tank which may be of 60,000 to 500,000 gallon capacity. At the time the final fermentor is being filled a culture from a 24-hour-old fermentation tank is also added to the fermentor. There are two ways of filling the final fermentor. One is to add the culture and partially fill the fermentation tank with sterilized corn mash, then after a period of a few hours add the rest of the mash. This method is known as a "deferred filling". The other and most commonly applied method is to fill the tank in one filling, adding the culture at the start. The advantage of using a "deferred filling" is that the culture multiplies many-fold before the rest of the mash is added, thus speeding up the final fermentation and tending to suppress contaminants.

The handling of the culture or seed is very critical. The plant is dependent on the bacteriologist's having present an absolutely pure stock culture of the bacteria in the spore stage. These spores are maintained on the standard soil-sand-carbonate mixture. The first step is to remove some of these pure-culture spores and mix them with a fresh sterilized corn or potato mash of

about 4 per cent starch concentration. This tube is then subjected to a heat shock or pasteurization treatment; for example, the tube may be placed in a boiling water bath at 212 F and held there for 90 seconds, then removed and cooled to 98 F. This tube is then known as the first laboratory stage. The heat shock is given to eliminate any vegetative cells or weak spores present in the tube and to stimulate germination of the remaining spores. The heat shock varies, depending on the size and nature of the tube and the character of the medium employed.

After heat shocking, the tube is placed in a constant temperature incubator at 98 F and held for 20 to 24 hours. During this time the spores have germinated and the vegetative cells or active form of the fermentation appear. At the end of 20 to 24 hours, the entire tube culture is transferred aseptically to a 1000 ml Erlenmeyer flask containing 600 ml of a sterile corn meal mash of 8 per cent concentration. After transfer, the first stage is plated out to detect if any facultative anaerobes such as lactic acid bacteria are present, and the second-stage flask is allowed to incubate for 24 hours at 98 F. At the end of this time, the second-stage flask is used to inoculate (3 per cent inoculum) an Erlenmeyer flask containing 3000 ml of a sterile 8 per cent corn meal mash and this flask is incubated for 24 hours at 98 F. The second-stage flask is also plated out to detect whether facultative anaerobes are present. After incubation of the large Erlenmeyer flask for 24 hours at 98 F, it is ready for inoculation into the first plant stage; providing, of course, that no contamination has shown up in the earlier stages. It may also be to one's advantage to carry out several more laboratory transfers before using it in the plant stage (see table 1 for typical data on the effect of transfer on yields of products).

The first plant stage consists commonly of a 5000 gallon jacketed, iron fermentation tank equipped with an agitator and inoculating device. The entire contents of the large Erlenmeyer flask are inoculated into this plant tank which contains about 4000 gallons of a sterilized 8 per cent corn mash which has been cooled to 98 F. After inoculation, the tank is agitated to mix the culture and mash and the tank is held under 15 pounds of sterile air or fermentation gas pressure to maintain an anaerobic condition and to prevent outside contamination from entering. The culture used to inoculate the first plant stage is also plated out to check for micro aerophilic or facultative anaerobic contamination. If for any reason any one of the previous stages shows contamination, the plant stage will not be used. A close watch is maintained at hourly intervals on the first plant stage and samples are plated out and tested at various intervals for gas formation, pH, acidity and temperature. The titratable acidity starts out low and builds up to a peak in approximately 18 hours; then, in a normal fermentation, starts to fall off. This point is known as the "break". As the plant stage "breaks" it is used to inoculate the final fermentation tank of a capacity of 60,000 to 500,000 gallons. These final fermentation tanks are usually of a pressure type withstanding 15 to 25 pounds of steam pressure and usually are either cylindrical, spherical or spheroid in shape. The tanks are fitted with gauge glasses, temperature recorders, vacuum breakers, safety relief valves and a manhole cover for use in cleaning. Various percentages of inoculum are used ranging from 0.5 to 3.0 per cent. In the final tank the fermentation is carried through to completion, utilizing a corn meal mash of 8 to 10 per cent concentration. Observation of the changes occurring in the corn mash shows the organism's amylolytic activity, as indicated by the decrease in viscosity and opaqueness of the mash. In addition to these changes, there is a vigorous gas production and, usually, a compact head formation consisting of protein, fibrous material and slime. This head is forced to the top of the liquid in the early stages of the fermentation and remains there until the fermentation is completed, at which time it tends to settle to the bottom. Some culture strains rarely head up.

TABLE 1. *Effect on the total solvents and acetone of transferring* Clostridium acetobutylicum *through 12 transfers on 8 per cent corn mash*

TRANSFER	TOTAL SOLVENTS	ACETONE PER CENT OF TOTAL SOLVENTS
	g/l	
2	15.08	35.45
3	15.58	29.90
4	16.03	32.40
5	16.62	30.80
6	18.40	28.00
7	18.53	29.80
8	19.30	30.50
9	19.16	29.50
10	19.98	28.50
11	19.99	25.00
12	19.85	25.80

A study of the cyclical changes in the development of *C. acetobutylicum* in the fermentation was made by Peterson and Fred (1932).

A very close watch is kept on the final fermentation tanks for changes in pH, acidity, gas formation and bacterial contamination (both microscopic, nutrient broth turbidity tubes and by means of plating). When the fermentation is completed (50 to 60 hours) the contents of the tank are pumped into a surge tank or beer well and the fermentor tank is cleaned, sterilized and made ready for the next fermentation. Sometimes it is advisable to pump the partially finished fermentation mash (at 20 hours or after the acid break) into a finishing tank in which the fermentation may be com-

pleted. The advantage gained here is that the finishing tank does not have to be sterilized and may be a large open-top tank. This procedure makes the pressure fermenting vessels available for re-use in a very short time and a large production schedule can be maintained. The one disadvantage is that the finishing tanks are never sterile and the contamination present may slighty affect the over-all yield and, at the same time, cause the acidity to rise in the mash. The final fermentor is the source of income and has to be handled economically. It usually contains $1,000 to $5,000 worth of raw materials and it is essential to maintain conditions necessary for the complete fermentation and recovery of all products. Figure 1 shows a typical modern flow diagram of the acetone - butanol fermentation of starches. Figure 2 shows Hortonspheres of 15,000 bbl capacity such as are now used in the acetone-butanol fermentation. In table 2, a comparison of acetone-butanol solvent production utilizing various grains is shown. Figure 3 shows typical colonies of strain B9 of *Clostridium acetobutylicum* grown on malt agar.

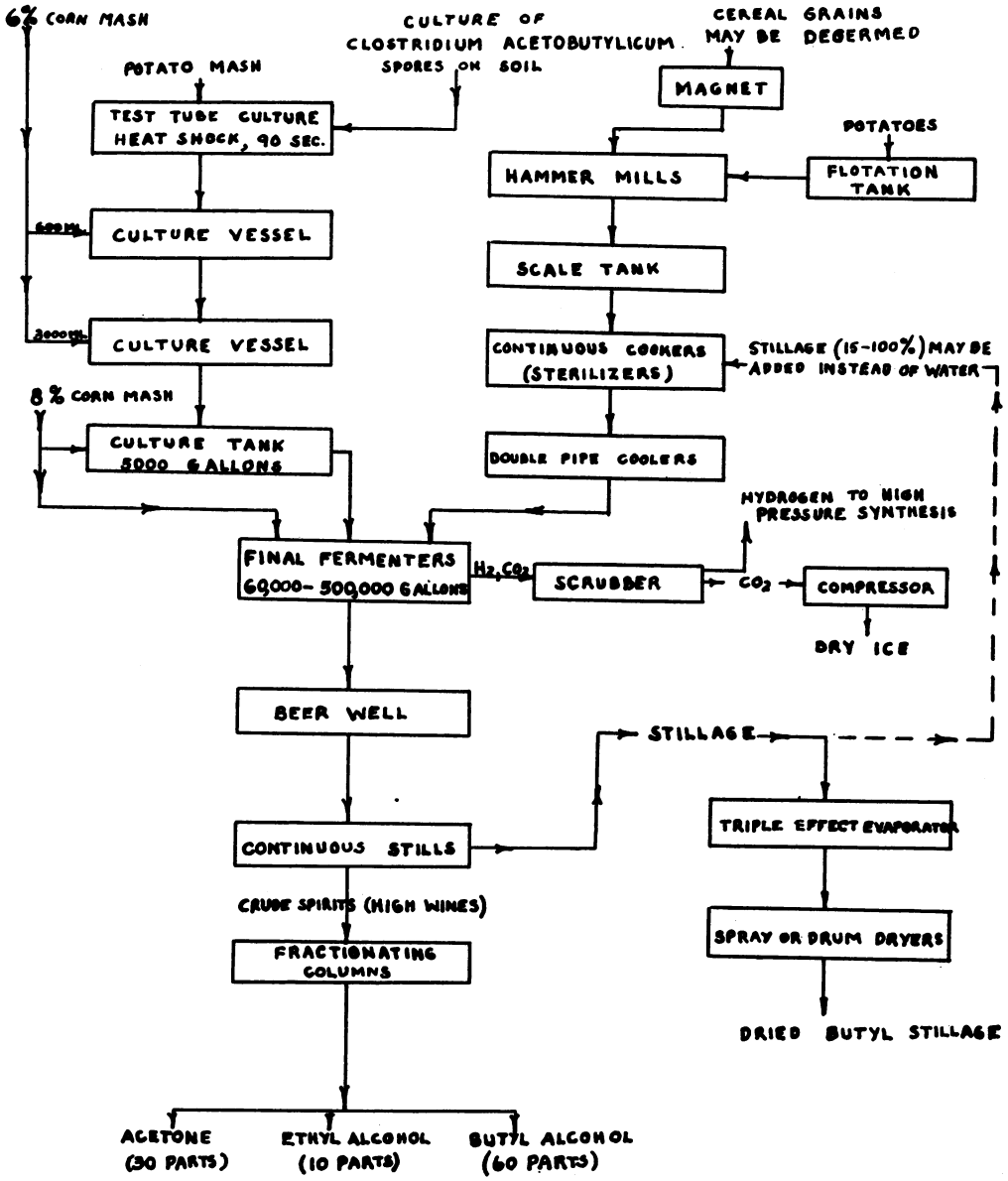
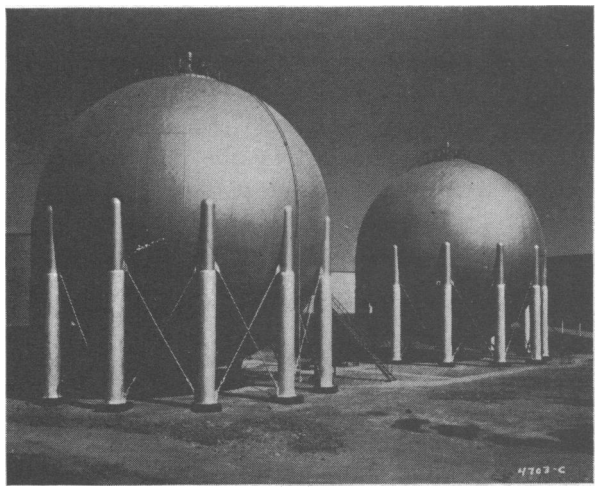


FIG. 1. Flow diagram of typical modern acetone-butanol fermentation using starch products.

Conditions For Fermentation

The optimum temperature for the acetone-butanol fermentation using *C. acetobutylicum* is 98 F. A range of 93 to 106 F results in a fairly normal fermentation, but with changes in solvent ratios and losses of acetone at the higher temperature unless the gases are scrubbed to remove the solvents. The organism produces its best yields under strict anaerobic conditions. Usually in open vessel fermentations this anaerobiosis is obtained by the formation of a head and presence of carbon dioxide over the surface of the mash. The pH may

range from 4.0 to 7.0 by the butanol organisms. The usual starting pH is 6.0 to 6.5 and the final pH, 4.2 to 4.4. The addition of calcium carbonate to the acetone-butanol fermentation medium causes a decrease in the yield of acetone and butanol in proportion to the amount of carbonate added; the volatile acids, butyric and acetic, are increased, while alcohol formation is



Photograph—Chicago Bridge and Iron Co., Chicago, Ill.

FIG. 2. Hortonspheres of 4,500 to 9,000 barrel capacity are now employed in the Acetone Butanol fermentation.

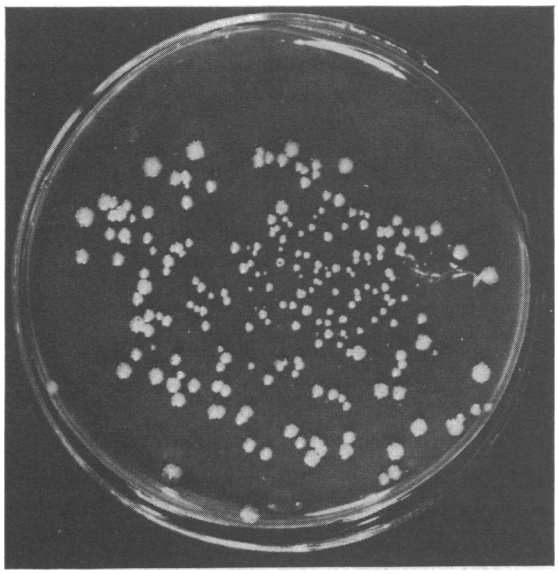


FIG. 3. *Clostridium acetobutylicum* (Weizmann) colonies, Strain B9 grown on malt agar.

suppressed. The addition of calcium carbonate to special grain mashes for the production of large amounts of riboflavin by use of *C. acetobutylicum* is the subject of a patent by Yamasaki (1942). The exact concentration of raw material to be used depends on the starch content. Usually, in plant practice, concentrations of corn of 8 to 10 per cent (5 to 6.5 per cent starch) are used and are readily fermented with high yields. Some materials, due to their high viscosity (potatoes, for example), cannot properly be handled when high concentrations are run.

NITROGEN REQUIREMENTS

The question of nutritional requirements for *C. acetobutylicum* has received considerable attention from numerous investigators. Oxford, Lampen and Peterson (1940), and Weizmann (1945), have found biotin and p-amino benzoic acid, respectively, to be the chief growth factors necessary for growth and fermentation by *C. acetobutylicum*. Actually, in plant practice, using ground wheat, corn, milo or rye, all the necessary accessory or stimulating substances are present, giving a normal fermentation. However, the addition of either ethyl grain stillage or butyl grain stillage results in a better than normal fermentation. Patents have been issued to Legg and Stiles (1936), Hancock (1934) and McCutchan and Arzberger (1935), using various amounts of stillage and using a continuous recycling

TABLE 2. *Comparison of acetone-butanol solvent production using various grains*

FERMENTATION MASH	TOTAL SOLVENTS	ACETONE OF TOTAL SOLVENTS	SOLVENT YIELD	
			Dry grain basis	Starch basis
	g/l	%	%	%
7% corn	15.76	28.03	25.30	35.00
8% corn	17.80	32.30	25.00	33.40
9% corn	19.93	31.60	25.50	34.00
7% wheat	13.62	29.67	21.50	30.00
7% granular flour	16.04	31.61	24.90	33.40
7% milo flour	16.84	28.67	28.30	35.20
7% whole milo	15.05	29.00	24.80	33.40

Culture used: *C. acetobutylicum* (Strain 16)

process. These processes not only give marked improvement in yield but are also of considerable economic value in that water and steam are saved. The process also reduces foaming in fermentors, making it possible to use larger volumes of mash in the tanks. The increases in yield are probably due to soluble proteins, proteoses, peptones, amino acids and vitamins present in the stillage which exert a stimulating effect.

CONTAMINATION PROBLEMS

The greatest problem in the fermentation industry is maintenance of sterile conditions completely through the plant's system of tanks, pipes, valves, cookers, coolers and other mechanical devices with which the mash comes in contact. These sterile conditions are not only necessary in the equipment but are also necessary in the cooked mash, and in the pure culture system from the original stock culture through the final fermentation. In maintenance of plant equipment it is necessary to put all tanks, valves, pipes, cookers and coolers under steam or steam pressure before using.

Inoculating lines are kept under steam continuously except for the time when they are used. Packing glands on agitators are usually filled with a disinfecting grease or supplied with steam seals. The valves on all connections are closely watched and replaced whenever necessary. In the handling of some types of materials like potatoes, considerable sand and dirt are encountered which clog lines, cause wear on valves and, in general, complicate the activities of the plant personnel.

Various types of contaminants have been found in the acetone-butanol fermentation. These contaminants find their way into the system in various ways such as through leaky coolers, dirty cookers, improper cooking, inadequate sterilization of the lines, valves, pumps, leaky equipment, use of a low temperature dilution water and backing up of the gas headers. Contaminants may be divided into three types. The lactic acid bacteria are the most common contaminants and cause of loss in yield. These lactic acid-producing bacteria have been the subject of papers by Thaysen (1921), Speakman and Phillips (1924), Fred, Peterson and Mulvania (1926) and Bekhtereva (1943). The microorganisms, *Lactobacillus leichmannii* (also called *Bacillus volutans*), a high acid-forming organism, *Lactobacillus buchneri* (*Lactobacillus mannitopoeus*), *Lactobacillus gracile* (*Leuconostoc*?) and *Lactobacillus fermenti* (*Lactobacillus intermedium*), are listed in the order of their decreasing inhibiting effect. The growth of *C. acetobutylicum* favors development of these bacteria: (1) By hydrolysis of the starch to fermentable sugar and (2) by proteolysis of the nitrogen compounds to amino acids. *Bacillus volutans* (or *Lactobacillus leichmannii*) is a gram positive organism possessing volutin granules that may be stained by methylene blue to a deep purple. The other species are long or short rods, some so small as to resemble cocci and diplo-cocci. These other species are not so harmful as *Bacillus volutans* (figures 4 and 5).

In the presence of certain strains of lactic acid bacteria the characteristic gas and head of the butyl alcohol fermentation are lacking and most of the mash remains as an unfermented residue in the bottom of the tank. These bacteria produce toxic amounts of lactic acid in the presence of *C. acetobutylicum* and which inhibit the conversion of the acetic and butyric acids to solvents, thus still further lowering the pH of the medium. Sometimes the plant fermentation may be saved or partially salvaged by the rapid addition of alkali to raise the pH to the normal level. Freiberg (1925) claimed that acid formation may be inhibited by rapidly decreasing the temperature to 85 to 95 F.

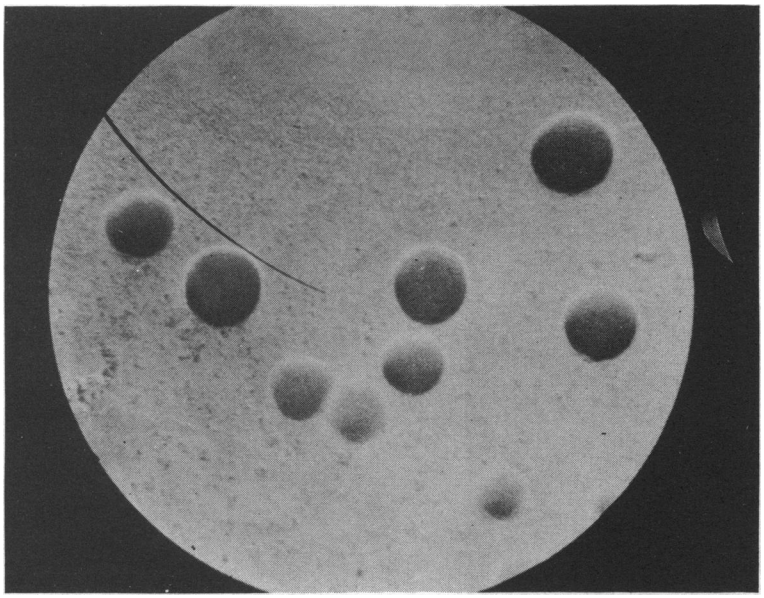


FIG. 4. *Lactobacillus* colonies 600×. These bacteria slightly affect the fermentation yield.

Weyer (1928) claimed the addition of butyl resorcinol or butyl phenol is toxic to the contaminants but not to the butyl organism. Methods of detecting contamination are gas evolution, titratable acidity, nutrient broth turbidity tube test, microscopic observation and plating. Marked changes in the pH or gas evolution rate indicate serious conditions in the fermenting mash. A second type of contamination is that caused by other bacteria or yeasts. Types such as *Streptococcus lactis*, *Bacillus subtilis* morphotype *globigii*, *Bacillus subtilis* morphotype *mesentericus*, and various yeasts have been found but their presence has been only slightly detrimental to the fermentation. The last type of contamination found and the most deadly and insidious of all types is the bacteriophage, or filterable virus. How the bacteriophage enters the fermentation process is a matter of wide speculation. Numerous theories have been developed, none of which has been proven. The bac-

teriophages affecting the acetone-butanol organisms have been found in the soil, in rivers, in the air and in some of the raw materials used. In other words, it is widely dispersed. The presence of a bacteriophage usually becomes evident at approximately the 18th hour after inoculation. The fermentation at this time is nearing the heading point when it is suddenly struck down and almost ceases to give off gas. The partial head and all the grain on the surface starts to settle, leaving a cloudy supernatant liquid which clears on standing. The total acidity at this time is usually 5.5 ml of 0.1 N NaOH per 10 ml of mash, which is about the normal peak acidity before the break. Upon being infected with a bacteriophage the acidity rises further and then remains constant. The bacteriophage can be isolated from the mash by adjusting the pH to 6.9 to 7.0 with NaOH and filtering through a Berkefeld or Mandler filter. These bacteriophages retain their virulence for an indefinite period at room temperature and they have been kept for years sealed off at reduced temperatures. Five minutes' exposure in a tube to live steam causes a precipitation of matter, probably protein, and the bacteriophage is inactivated. In table 3, data are presented showing the effect of a bacteriophage, which the author isolated, on solvent yields of *C. acetobutylicum*.

The only immediate recourse to a bacteriophage attack is to substitute at once an immune strain. Most industrial fermentation plants keep on hand a large number of strains, new isolations and previously immunized organisms which can be tested rapidly and substituted in the plant for the organism which has been affected. Various methods of selecting immune strains of the organism have been tried. Some methods have been patented (Legg (1928) and Legg and Walton (1938)) showing a process for selecting immune strains by repeated subculturing of the organism in the presence of small amounts of the bacteriophage. Others have found that by plating out the original strain and selecting various colonies they may isolate immune strains or, by placing a non-immune culture on a plot of open, unsterile soil and re-isolating the culture six months later, they may have an immune strain. The last procedure is the subject of a patent by Hanson (1937).

It has been the author's experience that any culture which has been properly immunized by suitable means, loses some of its activity. The fermentation is slightly slower, a somewhat lower concentration of mash is fermented and the solvent yield is slightly less than was previously obtained by the non-immune strain. An example of this is shown in table 4. The decreased yield is usually sacrificed, however, by the insistent demand for an immune culture since when a bacteriophage shows up in the plant, it usually spreads rapidly regardless of attempted controls and the only hope is for an immune strain or a complete change-over of raw material and cultures. The author has found that the bacteriophage, when first isolated, is superior for use in immunization treatments since this first filtrate does not have the potency of later filtrates. Potencies whereby the culture was affected by the addition of 1 to 5 billion dilution of a bacteriophage-containing filtrate are known to the author. During a siege of bacteriophage in the plant, the bacteriophage may become increasingly active to a point where the fermentation may not even start. McCoy, McDaniel and Sylvester (1944) isolated four serologically different strains of bacteriophage for the same butyl culture. They also describe a method of immunization.

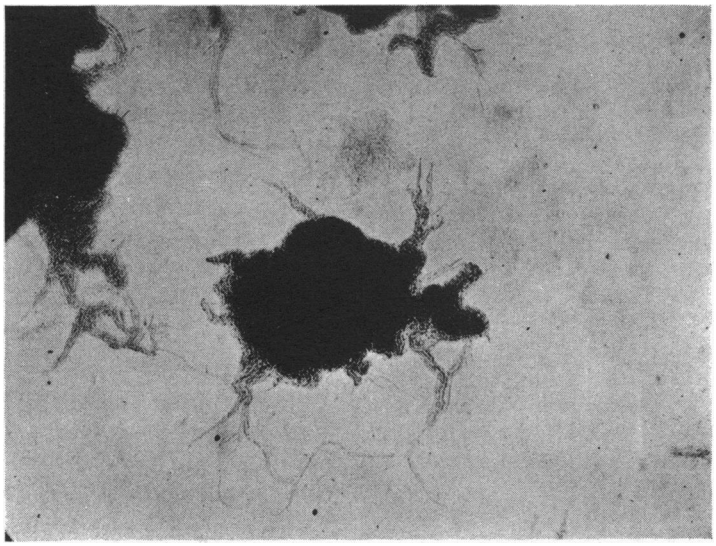


FIG. 5. *Lactobacillus* colonies 600×. These bacteria seriously affect the fermentation yield.

Toxic effects have been noticed in the fermentations when repeatedly re-cycling high concentrations of stillage or when using copper equipment for the first few times. After copper equipment has been used for some time a surface coating is built up which prevents the copper from acting as a poison. The acetone-butanol fermentation of corn by *C. acetobutylicum* is usually inhibited by 40 ppm of copper and almost completely inhibited by the addition of 50 ppm copper. The maximum tolerated limit lies between 30 and 40 ppm of copper ion. This limit varies, however, depending on the strain and whether it has become acclimated to high levels of copper.

TABLE 3. *Effect of bacteriophage on the acetone-butanol fermentation of corn mash by* Clostridium acetobutylicum *(Strain 16)**

FLASK	DILUTION OF BACTERIOPHAGE 1 ML ADDED	TOTAL SOLVENTS
		g/l
1†	control (none)	16.41
2	Undiluted	0.36
3	of 1-10 dilution	0.56
4	of 1-100 dilution	0.86
5	of 1-1,000 dilution	0.64
6	of 1-10,000 dilution	1.21
7	of 1-100,000 dilution	1.06
8	of 1-1 million dilution	15.82
9	of 1-10 million dilution	15.92
10	of 1-100 million dilution	15.74
11	of 1-1 billion dilution	16.49

* A 3 per cent inoculum was used.

† 8 per cent Corn mash of 700 ml volume was used in all flasks.

TABLE 4. *Comparison of yields of non-immune and immune cultures of* Clostridium acetobutylicum *in 7 per cent corn mash*

TOTAL SOLVENTS			YIELD	
Culture-strain	Grams per liter	Acetone of total solvents	Dry grain	Wet starch
		%	%	%
16*	15.66	32.30	24.90	33.10
16	16.04	30.76	25.53	34.00
16	16.04	31.30	25.53	34.00
16 I†	15.20	31.81	24.63	32.80
16 I	15.31	31.66	24.62	32.83
16 I	15.06	31.44	24.22	32.80

* Culture 16—Standard non-immune strain.

† Culture 16 I—Immune to bacteriophage.

YIELDS AND RECOVERY OF END PRODUCTS

The most important products formed in the acetone-butanol fermentation are n-butyl alcohol, acetone, ethyl alcohol, carbon dioxide, hydrogen and riboflavin-containing feeds from the fermentation residue. In the normal acetone-butanol fermentation of starches we may expect a ratio of solvents of approximately 60 butyl, 30 acetone and 10 ethyl. Various factors influence changes in these ratios. The material to be fermented, cultures used, temperature, use of stillage, contaminants present and the addition of various chemicals, all serve to change the ratio of solvents produced. Sucrose, levulose and xylose yield normal ratios of solvents but arabinose (a 5 carbon sugar) yields a 50–40–10 ratio. We can expect from 4.3 pounds of corn, 1 pound of mixed solvents from the *C. acetobutylicum* fermentation. This corresponds to a yield of 1 pound of mixed solvents from 2.9 pounds of starch. In table 5, there is compiled from laboratory data the yield of products normally obtained from corn mash fermented with *C. acetobutylicum*. In addition to the products previously mentioned, a yellow oil has been obtained by Marvel and Broderick (1925). The yellow oil constitutes 0.5 to 1.0 per cent of the total solvents and consists of a high boiling point mixture of n-butyl, amyl, iso-amyl and n-hexyl alcohol and the n-butyric, caprylic and capric esters of these alcohols.

The stillage obtained after distillation of the solvents has become a major by-product in the past ten years due to its widespread use in animal feeds. The normal dried stillage contains various vitamins, among them riboflavin or vitamin B_2 in amounts of 40 to 80 micrograms per gram (dry weight), and vitamin B_{12} in amounts of 20 to 30 milli micrograms. Various investigators have found ways to increase the amount of riboflavin produced in the acetone-butanol fermentation by *C. acetobutylicum*.

Miner (1940) first discovered that butyl stillage residues contain vitamin B_2 and that the various butyl bacilli are capable of synthesis of the vitamin. A short time later Yamasaki (1942) discovered that the addition of calcium carbonate to various cereal mashes causes an increase in riboflavin content. Arzberger (1936) found that iron present in the grain mash was responsible for the naturally low yield of the vitamin and that certain other metals such as Ni, Co, Cu, Pb and Zn affected the riboflavin synthesis. Upon removal of all such substances he obtained yields as high as 3,000 micrograms of riboflavin per gram of dry mash. Walton (1945) found that by use of brown rice mixed with corn, using Arzberger's modification, the amount of riboflavin could be increased to about 4,000 micrograms per gram and occasionally to as high as 6,000 micrograms. Legg and Beesch (1945) found that the addition of from 0.04 to 0.035 per cent of a substance capable of liberating a sulphite radical in the presence of water stabilized the production of riboflavin giving consistently higher yields. The author later found that by fermenting a granular wheat flour mash of 4 per cent concentration (relatively iron free) he could obtain riboflavin in amounts of 6,000 to 7,000 micrograms per gram. Table 6 shows typical data obtained. Leviton

(1946) theorized that the destruction of riboflavin by the *C. acetobutylicum* operates through a peroxide mechanism, which is supported by experiments in which significant increases in yield of riboflavin are obtained by use of sodium hydrosulfite and traces of crystalline catalase. Hickey (1947) has found that the iron present in the mash can also be inactivated by use of 2,2′-bipyridine which is well known for its ability to form a very stable complex with the ferrous iron. The author has also found that the presence of 40 ppm of 8-hydroxy-quinoline will inactivate 2–3 ppm of iron, resulting in a fermentation giving high yields of riboflavin. Other iron-forming complex salts such as o-phenanthroline and sodium catechol disulfonate were ineffective.

These various methods of riboflavin production on a commercial scale have been superseded by the use of cultures of *Eremothecium ashbyii* and *Ashbya gossypii* when grown on ethyl and butyl grain stillages and dextrose-stick liquor corn steep mashes, respectively.

TABLE 5. *Average yields obtained from acetone-butanol fermentation of corn with* Clostridium acetobutylicum

	STARCH	TOTAL SOLVENTS	BUTYL ALCOHOL	ACETONE	ETHYL ALCOHOL	HYDROGEN	CO_2
	lb	*lb*	*lb*	*lb*	*lb*	*lb*	*lb*
1 bushel corn (56 lbs) 12% moisture 63% starch	35.3	13.3	7.71	3.72	1.86	0.6	22.1
1,000 pounds starch	1,000	380	220	106	53	17	626
1,000 pounds corn 12% moisture 63% starch	630	238	138	66.6	33.3	10.7	394
1,000 gallons mash 9% wet basis corn	473	178	103	49.6	24.9	8.04	296

Yield on dry corn 27.0 per cent
Yield on wet corn 23.8 per cent (12 per cent moisture)
Yield on starch 38.0 per cent (63 per cent starch in corn)
Composition of solvents:
58 per cent n-butyl alcohol
28 per cent acetone
14 per cent ethanol

GASES

The gases, carbon dioxide and hydrogen, produced during the fermentation, weigh over 1.5 times as much as the neutral solvents obtained. From the start of the fermentation until the 15th hour the volume of hydrogen given off is much greater than the carbon dioxide, due in part to the carbon dioxide being absorbed in the mash in the early hours. The total gas evolved contains about 60 per cent carbon dioxide and 40 per cent hydrogen by volume. Table 5 gives the average yield of gases obtained from various amounts of corn and starch. The large quantities of mixed hydrogen and carbon dioxide produced are of a high purity and their utilization has been the subject of intensive research. The separation of CO_2 from H_2 is done by passing the gas mixture under pressure through a scrubbing tower which dissolves almost all of the CO_2, leaving the hydrogen. The hydrogen is then given a treatment with caustic soda solution which results in a highly purified product. In this pure form the hydrogen may be used for synthesis of ammonia without difficulty and the nitrogen required may be easily obtained in satisfactory pure form by burning a part of this hydrogen in air. It is also possible by passing the mixed gases over heated carbon to procure a mixture of carbon monoxide and hydrogen which can serve as materials for the synthesis of methanol, if desired. If recovery of the gases is not made the gas is usually scrubbed and approximately 1 per cent of the solvents is recovered in the ratio of 65 per cent acetone, 30 per cent butanol and 5 per cent ethanol.

TABLE 6. *Production of high yields of riboflavin from 4 per cent granular wheat flour fermentations utilizing* Clostridium acetobutylicum (*Strain 38*)

FLASK NUMBER	TOTAL SOLVENTS	*RIBOFLAVIN MICROGRAMS PER GRAM OF DRIED FERMENTATION RESIDUE
	g/l	
1	8.90	5400
2	8.74	4700
3	8.71	6790
4	8.92	6995
5	8.88	6500

* Average results of three flasks.

BY-PRODUCT FEEDS AND FEED VALUES FROM THE ACETONE-BUTANOL FERMENTATION OF AMYLACEOUS MATERIALS

The disposal of by-products and wastes from a fermentation industry is an ever-present problem. Certain by-products such as distiller's grains have considerable value in animal feeds. The advances made in our knowledge of vitamins have supported the feeding of the distillery by-products to various animals. Stillage from the acetone-butanol plants previously was run into the river as it was the most economical procedure. Recently, the distilleries have been compelled to process their wastes in order to dispose of them without excessive pollution of the streams even though this processing was done at a loss. Research on ways to utilize these

TABLE 7. *Feed values recoverable from acetone-butanol fermentation of blackstrap and invert molasses*

FRACTIONS OF STILLAGE	KIND OF MOLASSES	DRY MATTER IN STILLAGE	100 POUNDS MOLASSES GIVES POUNDS OF		ANALYSIS DRY BASIS			RIBOFLAVIN MICROGRAMS PER GRAM
			Dry feed	Protein	Protein	Fat	Ash	
		%			%	%	%	
Total solids evaporation whole stillage...	Invert	1.15	17.7	6.5	36.8	0.31	12.1	52
	Blackstrap	2.71	28.6	6.0	20.8	0.14	23.2	38
Solids recoverable by centrifuge.....	Invert	0.17	2.6	2.0	77.6	2.00	4.8	49
	Blackstrap	0.22	2.3	1.3	57.5	2.60	7.2	27
Dried effluent from centrifuge.....	Invert	0.96	14.8	4.1	28.1	0.0	13.4	54
	Blackstrap	2.50	26.3	4.2	16.0	0.0	25.9	37

TABLE 8. *Acetone-butanol fermentation—yields of solvents, feed, protein, riboflavin*

100 POUND	*TOTAL SOLVENTS	BUTYL ALCOHOL	ACETONE	ETHYL ALCOHOL	DRY FEED	PROTEIN	PROTEIN	MICROGRAMS PER GRAM RIBOFLAVIN
					lb	*lb*	%	
Dry corn.....	25 to 26	14.3 to 14.9	7.3 to 7.6	0.35 to 0.36	36.8	10.8	32.8	40 to 70
Dry hard winter wheat.....	24 to 25	13.7 to 14.3	7.0 to 7.3	0.34 to 0.34	35.6	14.3	40.3	40 to 70
Dry rye.....	22 to 24	12.6 to 13.7	6.4 to 7.0	0.31 to 0.34	35.0	14.1	40.0	30 to 50
Blackstrap molasses (58% sugar)...	16 to 17	11 to 12	4.9 to 5.0	0.05	28.6	6.0	20.9	30 to 50
Invert molasses (78% sugar).....	23 to 24	15.5 to 16.0	6.5 to 7.0	0.07	17.7	6.5	36.8	50 to 65

* Assume butanol-acetone-ethanol proportion of 57-29-14 for grain and 68-29-3 for molasses.

TABLE 9. *Feed values recoverable from acetone-butanol fermentation of whole ground corn, wheat and rye*

FRACTIONS OF STILLAGE	*KIND OF GRAIN	DRY MATTER IN STILLAGE	100 POUNDS DRY GRAIN GIVES		ANALYSIS DRY BASIS STILLAGE				RIBOFLAVIN MICROGRAMS PER GRAM
			Dry feed	Protein	Protein	Fat	Fibre	Ash	
		%	*lb*	*lb*	%	%	%	%	
A. Solids by evaporation of whole stillage.....	Corn	2.32	36.8	10.80	32.8	14.2	4.9	4.1	41
	Wheat	2.54	35.6	14.32	40.3	6.5	6.8	5.5	47
	Rye	2.46	35.2	14.10	40.0	6.6	5.3	6.9	35
B. Dried solids on 40 mesh screen.....	Corn	0.34	8.6	1.00	19.1	16.9	16.6	1.5	6
	Wheat	0.60	8.4	1.59	18.9	10.2	21.3	1.4	14
	Rye	0.74	10.6	2.06	19.4	5.7	4.3	1.8	26
C. Solids thru 40 mesh screen.....	Corn	1.95	31.0	9.20	38.8	11.1	1.5	5.2	46
	Wheat	2.02	28.3	14.31	50.6	4.1	2.3	7.0	40
	Rye	2.04	29.2	13.42	46.0	5.7	1.1	7.8	35
D. Solids centrifuged from C.....	Corn	0.31	4.9	1.36	22.9	10.8	5.3	1.6	16
	Wheat	0.38	5.0	2.06	35.0	10.0	6.1	2.1	35
	Rye	0.35	5.0	1.90	32.1	13.0	3.6	2.3	36
E. Dried effluent from centrifuge.....	Corn	1.68	26.6	7.85	42.1	11.4	0.4	6.4	31
	Wheat	1.39	19.5	4.96	30.0	10.3	8.7	0.3	42
	Rye	1.69	24.2	10.22	49.6	13.4	0.5	13.2	36

* Concentration of corn in original mash 6.3 per cent; wheat 7.1 per cent and rye 7.0 per cent

TABLE 10. *Yield of screenables and solubles in stillage from acetone-butanol fermentation of potatoes*

100 pounds	Whole potato of 78–84 per cent moisture gives approximately: 0.5–0.52 pounds of dry screenables and 4.5–4.7 pounds of dry solubles.
100 pounds	Dry potato gives approximately: 2.4–3.2 pounds of dry screenables and 2.2–2.9 pounds of dry solubles; plus 19.8 pounds of total mixed solvents.

stillages thus was stimulated. Evaporation and incineration of the concentrates produce a fertilizer rich in potash but not sufficiently valuable to pay the processing costs.

Miner (1940) found that such still residues were valuable as sources of vitamin B_2 and protein in poultry and animal feeds. Groschke and Bird (1941) and Braude and Foot (1942) also found that the stillage residues of the acetone-butanol fermentation of molasses and grains

possess a high nutritional value as feed for livestock. Since that time thousands of tons of feeds have been furnished as by-products of the acetone-butanol fermentation of starches and sugars. Tables 7, 8, 9 and 10 contain data compiled by the author, showing the various amounts of protein, vitamins, et cetera, contained in various types of acetone-butanol stillages.

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