THE UTILIZATION OF WASTE MOLASSES IN THE PRODUCTION OF

I. ACETONE AND BUTANOL
II. NORMAL BUTYRIC ACID

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INTRODUCTION

The industrial world has felt the influence of fermentation processes, especially during the past thirty years; and is beginning to realize the tremendous significance of the application of industrial microbiology in the development of useful chemicals from cellulose, starch and sugars.

The microbiological flora of the tropics is rich in organisms capable of industrial utilization, and the industry of the sugar cane offers abundant and inexpensive material, that may become the basis of great future industries. In the writer's opinion, carbo-hydrate nations are destined to exercise a controlling role in human affairs through chemical synthesis and fermentation processes.

This paper treats of two such processes, and will be divided in two parts; the first dealing with the production of Acetone and Butanol from waste molasses, and the second with that of normal butyric acid from the same source. While the first part embodies the account of a finished piece of industrial research (in so far as laboratory work is concerned) that was started during July, 1931; the second presents the progress made to date in a new investigation initiated only a few months ago. This fact will account for the lack of definite data in the subject matter of part two.

PART I

BUTANOL AND ACETONE FROM WASTE MOLASSES

The Necessity of Industrial Research in the Industry of the Sugar Cane.—While there exists no single great industry more needful of industrial research than that of the sugar cane, there is none showing less interest and progress in this respect. There have existed several reasons for this attitude, a discussion of which lies beyond the scope

of this article. But whatever reasons existed in the past for this apathetic attitude towards industrial research, they are no longer tenable under the existing general conditions of this great industry. Millions are being wasted every year in the form of unutilized by-products and waste materials while the price of the main product drops to unprofitable levels. It seems that those responsible for the wastes are so close to them that they have come to regard them as unavoidable accompaniment of the industry. Meanwhile the dextrose industry has become a serious competitor, and the commercial production of Levulose promises a still stronger future rival. At the same time the technical world hears of sugar from wood waste in Sweden and Germany, and sugar again, from potatoes in Ireland. Is the cane-sugar industry to remain in the worn-out traditional channels, blind and oblivious to the "New Era" that will revolutionize the world through carbohydrate chemistry?

Possibility of Producing Butanol and Acetone from Waste Molasses.—With a determined purpose of doing his share (no matter how small and inadequate) for the amelioration of this existing trend of affairs, and with a deep rooted conviction that if the sugar industry is going to servive its present crisis, new products must be manufactured either from sugar itself, or its by-products, the writer started his investigation on the problem of the fermentation of final molasses for the production of butanol and acetone. From his search of the literature the writer knew of no existing fermentation process of commercial magnitude for the production of these solvents from Waste Molasses, at the time of the commencement of his investigation, July 1931.

Searching for the Fermenting Organism.—Having learned from the literature on this subject that the organisms responsible for the acetone-butylic fermentation of carbohydrate material were widely distributed in nature, the writer decided to try as possible sources such things as soil, decaying vegetable matter, potatoes, sweet potatoes, beets, and sugar cane. For the culture medium, a dilute solution of final molasses in water was selected, with such chemical and physical modifications as would best suit the growing and development of the given organism and its power for the production of solvents. The word "Solvents" is used throughout this work to include all the valuable liquid products of fermentation.

The molasses solution was placed in test tubes of about 50 ml. capacity, and inoculated in each case directly with small pieces of the material suspected of containing the butyl bacillus. After incuba-

tion for a period of from 36 to 48 hours, the presence of butanol was to be detected first hand, by that most chemical of human senses, the sense of smell. If the characteristic butanol scent could be detected, then further work was to be done to isolate the organism and set it to work under conditions favorable to its growth, development and solvents producing power.

The preliminary work as described above was conducted without success in the great majority of cases; the potatoes, sweet potatoes, and the soils in which they grew giving negative results. The writer reached the conclusion that either these materials did not contain the butyl bacillus, or else, the strains contained in any or all of them could not be grown and developed in the culture medium selected for this work.

The search for the organism was then started among the sugar cane varieties grown in Puerto Rico; and this time luck came to help out the writer's labors. Not less than 50 different varieties and the soil around their roots were examined, and in a little over 20 per cent of these, the organism was found. However, not all of them showed the same characteristics, ability to grow, develop and produce the required solvents in the chosen medium. The apparently most promising strains were obtained from the following canes:—POJ–2725; 2883; 2873; 979; 1228; FC–588; 916; 998; and PR–820 and 807. But the only one that convinced the writer of possessing very remarkable power for doing the work, was a strain obtained from the roots of a cane of the Kassoer variety.

Having thus obtained promising material, the writer proceeded to the work of purification and isolation of the bacillus. This work will be described in the next paragraph.

Isolation of the Bacillus in Pure Culture.—The bacillus found on the roots of the Kassoer cane was isolated as follows: A set of 12 test tubes each containing 10 ml. of sterile mash of 4 deg. Brix density, were inoculated with small amounts of the unsterilized cane roots. The tubes were then immersed in boiling water for 50 seconds and immediately cooled in running water. They were then incubated for 36 hours and examined for odor and gas production. The tube showing most vigorous fermentation and strongest butyl odor was chosen, and further incubated until fermentation was completed; reheated in boiling water, cooled, and the contents plated in malt gelatine agar. After inoculation of the malt gelatine agar, the medium was poured into the inverted lid of a sterile Patri dish, and the bottom section of the dish was then floated on the liquid. After incubation the two

parts of the plate were easily separated and the colonies fished. These colonies were used for further propagation as found necessary.

Adapting the Medium to the Bacillus.—Lack of inverting power and inability to attack sucrose for the production of solvents were soon discovered in the bacillus. The bacillus, on the other hand, readily attacked and decomposed reducing sugars with the production of the desired solvents. Hence the sucrose in the molasses used for making up mashes was always inverted before inoculation.

After some experimental work details of which would occupy too much space, the conclusion was reached to make the mashes according to the procedure described below, calling this mash "The Standard Mash".

Ninety grams final molasses were weighed into a two liter Erlenmeyer flask, four hundred ml. distilled water added and the molasses dissolved by heating on a water bath with occasional shaking. When solution was effected, 1.5 ml. strong sulphuric acid were added, and the flask was autoclaved during half hour at 20 pounds pressure to effect inversion. After cooling to about 50 deg. C., five and a half grams of calcium carbonate, and 1.5 ml. strong ammonia water were added to the mash in the flask. When effervescence subsided, the mash was completed to 1,800 ml. with distilled water, plugged with non-absorbent cotton, and again autoclaved for half hour at 20 pounds pressure. When thus prepared, this standard mash gave a reading of 90 mv. at the potentiometer, equivalent to pH–6. Any small variation from a potentiometer reading of 90 mv. was corrected by addition of hundredth normal sulphuric acid or sodium hydroxide; as found necessary. The bacillus worked very satisfactorily in this medium.

Fermentation Tests.—Many fermentation tests using the pure culture obtained and the standard mash prepared as described above were conducted at various temperatures, ranging from 30 to 40 deg. C. to ascertain the optimum temperature of fermentation. Results of those tests showed that the optimum temperature for a successful fermentation was 35 deg. C. At this temperature the greatest yield of solvents was obtained, fermentation being completed in from 48 to 54 hours after its initiation. When working at temperatures above 35 deg., C. it was found that fermentation was rapid and vigorous; but the yield of total solvents was less than when working at 35 deg. C., while the ratio of acetone to butanol was higher. At 39 deg. C. the organism worked very briskly for some time but all action soon ceased, and when examined under the microscope the bacilli had ap-

parently lost their characteristic motility, very few vegetative cells being observed. When temperatures from 30 to 35 deg. C. were used the fermentation was rather sluggish, and instead of from 48 to 54 hours, it took from 60 to 72 hours to complete it. The table on next page shows the influence of temperature during the fermenting period.

TABLE SHOWING THE EFFECT OF TEMPERATURE DURING FERMENTATION

% T. S. = PERCENT TOTAL SOLVENTS

Test No.	Temperature of Fermentation Deg. C.	Time of Active Fermentation Hours	Analysis of Products				Ratio of Butanol to Acetone	
			T. S. %	Butanol %	Acetone %	Ethanol %		
1.....	30	70	23.50	17.82	4.95	0.73	3.6	I
2.....	31	66	24.05	18.00	5.00	1.05	3.6	I
3.....	32	63	24.50	18.28	5.07	1.15	3.6	I
4.....	33	60	25.80	19.31	5.51	0.98	3.5	I
5.....	34	57	27.10	20.27	5.78	1.05	3.5	I
6.....	35	49	29.50	21.98	6.27	1.27	3.5	I
7.....	36	45	27.65	20.07	6.08	1.50	3.3	I
8.....	37	38	24.49	17.54	5.68	1.28	3.1	I
9.....	38	32	18.90	12.98	4.99	0 93	2.6	I
10	39	25	12.00	7.57	3.78	0.65	2.0	I

The above figures show that the optimum temperature for fermentation is 35 deg. C.; that below this temperature fair results may be obtained down to about 32 deg. C. and up to about 37 deg. C. Temperatures above 37 deg. C. give very poor results as to total yields and butyl alcohol-acetone ratio; while temperatures below 32 deg. C. though not giving very bad yields, take too long to finish fermentation.

Acidity of Mashes.—The acidity of all mashes was controlled by pH determinations using the potentiometer and quinhydrone electrode. Having found that the bacillus worked best under slightly acid conditions, the initial pH of all mashes was regulated to a reading of 90 mv. as stated previously when describing the "STANDARD MASH".

An experiment was run to determined the variations in acidity during and upon completion of fermentation.

During a normal fermentation at optimum temperature, it was observed that the mv. reading rose steadily as soon as fermentation started, continuing this rise during the first 22 or 24 hours, when a potentiometer reading of about 160 mv. could be observed. After this concentration of acidity was reached, the mv. readings started to yield descending values till a constant or nearly constant value of from 125 to 130 mv. was obtained which remained so until fermentation stopped.

In cases of a poor fermentation test due to contamination of the inoculum, or of the mash after inoculation, or to any other inhibiting force, the acidity continued to rise going up to readings between 190 and 210 mv. Every time that this happened, poor yields of solvents were to be expected; examination of a sample under the microscope showing less motility of the organism, signs of contamination; and in extreme cases the organism disappeared altogether from the field of vision.

Thus the determination of pH values periodically (say every 2 or 3 hours) proved to be an excellent control, as an abrupt rise in mv. readings was a sure indication of forecoming trouble. The following curves show the rise in pH during a (I) normal, vs. a poor (2) fermentation.

The following table offers some figures obtained from fermentation test conducted at optimum temperature using "Standard Mash".

DATA

Hour and date of inoculation ..... 10 A. M. Oct. 1, 1931
Hours after inoculation at which fermentation started ... 4 hours
Date of completion of fermentation ..... 3 P. M. Oct. 3, 1931
Hours of active fermentation 53
Incubating temperature ..... 35 deg. C.

Mash No.	Starting Bx.	Final Bx.	Starting Mv.	Final Mv.	S. G. of Distillate at $^{20}/_{4}$ C.	% T. S.	% Butanol	% Acetone
1.....	4.60	2.7	95	152	0.9940	20.55	15.88	4.55
2.....	4.70	2.7	93	155	0.9937	22.27	16.65	4.60
3.....	4.60	2.3	88	130	0.9923	30.70	22.64	6.40
4.....	4.60	2.5	90	153	0.9940	20.55	16.00	4.40
5.....	4.60	2.2	89	125	0.9922	31.36	22.76	6.60
6.....	4.70	2.4	87	132	0.9930	26.33	19.22	5.89
7.....	4.65	2.6	93	142	0.9935	23.20	17.59	4.35
8.....	4.60	2.6	89	151	0.9937	22.27	17.24	3.98
9.....	4.60	2.6	85	145	0.9938	21.82	16.42	3.87
10.....	4.60	2.4	86	135	0.9933	24.44	18.19	4.92

NOTE: — The percentage ethanol produced may be found by subtracting from % T. S. the sum of the percentages of butanol & acetone.

Determination of Optimum Sugar Concentration.—From an industrial point of view it was thought very important to determine the optimum sugar concentration in the mash at which the organism would work efficiently.

A series of 12 mashes varying in sugar concentration from 3.65 g. of total sugar to 11.25 grams per 100 ml. of mash were inoculated with the organism, using the same seed for all. After completion of the incubation period, the following results were obtained:

Mash No.	Concentration of T. Sugars Grams per 100 ml.	No. of hours to complete Fermentation	Temp. of Ferm. 35 deg. C.	% Total Solvents on wt. of sugars	% Acetone on wt. of sugars
1	3.65	48	"	24.75	5.21
2	4.25	48	"	27.74	6.09
3	4 55	50	"	27.30	6.12
4	4.85	50	"	27.12	6.22
5	5.14	52	"	22.85	5.20
6	5.45	60	"	10.10	2.50
7	5.75	66	"	7.39	2.00
8	6.05	72	"	4.13	1.15
9	7.25	80	"	1.72	0.65
10	8.50	No ferment...	.....	.....	.....
11	9.75	" "	.....	.....	.....
12	11.25	" "	.....	.....	.....

From a study of the data in the above table, it is apparent that best results are obained when working with sugar concentrations of from 4.25 to 4.85 grams per 100 ml. of mash. Fair results may be obtained with concentrations of 3.65 and 5.14 grams of sugar per 100 ml. of mash; but the commercial optimum would be attained when working with a concentration of 4.85 grams of sugar per 100 ml. of mash. With this concentration every 100 ml. of mash will yield on distilling 1.315 gram of total solvents or about 1.65 c. c. It may be observed that mashes Nos. 2 and 5 give slightly higher yields per sugar unit; but having lower concentrations, the actual weights of solvents obtained per 100 ml. of mash are smaller. Also the plant capacity for fermentation and distillation would have to be larger if these concentrations were used commercially. The yields in grams and ml. of solvents per 100 ml. of mash in the cases of the best three concentrations as represented by mashes Nos. 2, 3, and 4 follows:

Mash No.	Grams T. S. per 100 ml of mash	Ml. total solvents per 100 ml. of mash
2	1.18	1.48
3	1.24	1.55
4	1.315	1.65

Activating Agents.—Another fermentation test was run to find the effect of Lamp-Black and Kieselguhr when used in the preparation of the mash separately, and together, in varying proportions. The results obtained are shown in the table below:

TOTAL WEIGHT OF ALL MASHES: 1000 GRAMS

Mash No.	% T. Solids on Wt. of Mash	% T. Sugars on Wt. of Mash	Lamp-Black used Grams	Kieselguhr used Grams	Total Solvents	% Butanol
1	8.8	4.85			24.60	19.07
2	"	"	1		26.50	21.19
3	"	"	2		28.50	23.05
4	"	"	3		25.10	19.41
5	"	"	4		24.60	18.68
6	"	"	5		22.00	16.17
7	"	"		1	27.70	21.68
8	"	"		2	27.70	21 85
9	"	"		3	25.10	19.10
10	"	"		4	25.10	18.95
11	"	"		5	25.10	18.87
12	"	"	1	1	27.80	23.00
13	"	"	2	2	30.00	25.41
14	"	"	3	3	28.70	23.01
15	"	"	4	4	30.00	24.40
16	"	"	5	5	30.00	24.25

A perusal of the table on page 15 will reveal at a glance the beneficial effect obtained by the use of the activating agents discussed on that page. The effect is more striking when using both Lamp-Black and Kieselguhr together. In case when Lamp-Black is used alone, the maximum increase in yield of solvents is obtained by using 2 grams of this substance; and this increase amounts to 3.9 per cent on the weight of sugars in the mash or 15.86 per cent over the yield obtained when no activating agent is used. Using quantities much above this maximum give very little increase, no increase, or even deleterious results; for instance, when 5 grams lamp-black were used the yield of solvents was actually less than when using no activating agent at all. In the case of Kieselguhr alone the maximum increase was obtained when using either 1 or 2 grams of the substance; no further increase being obtained by using larger amounts. In this case the increase in yield amounts to 3.10 per cent on the weight of sugars, or 12.6 per cent over the yield obtained without using activating agents. A maximum yield is obtained when using both activating substances together in the proportion of two grams of each; and in this case the increase amounts to 5.40 per cent on the weight of sugars, or 21.81 per cent over results obtained when using no activating agents.

These results show that in a commercial process it would pay to use these activating substances, as the increase in yields are remarkable, especially when using both activating substances together.

Industrial Possibilities.—The writer's opinion is that the Bacillus ACETOBUTYLICUM discovered and isolated by him could have profitable industrial application; and this opinion is based on the following facts:

1. So far there exists no large scale production of these solvents using FINAL SUGAR HOUSE MOLASSES as the raw material.

2. Final molasses is to-day the cheapest carbohydrate source in the market, its price in Puerto Rico being about 3.5 cents a gallon, delivered at the various mills. A gallon of Puerto Rican final molasses contains an average of six pounds total sugars.

3. This bacillus is able to produce more yield of solvents per unit of sugar content of the fermenting medium than any other known so far.

4. The bacillus which is most widely used commercially to-day is that of WIEZMANN, and it produces butyl alcohol, acetone, and ethyl alcohol in the ratio 6 : 3 : 1, respectively using corn mash. This bacillus has not been able to produce these solvents profitable from mo-

lasses mash. The writer's bacillus produces these same solvents in the ratio 14:4:1, respectively, using final molasses mash of from 8 to 9 deg. Brix, corresponding to a sugar concentration of from 4.5 to 5.0 per cent.

5. The maximum actual laboratory yield obtained from 1 gallon FINAL MOLASSES by the writer has been:

Total solvents	30.00 per cent;	or 1.95 pounds
Butyl alcohol	22.31 per cent;	or 1.45 pounds
Acetone	6.15 per cent;	or 0.40 pounds
Ethyl alcohol	1.54 per cent;	or 0.10 pounds

The present average market value of the above products, per pound, are 10, 9, and 4 cents, respectively, for butyl alcohol, acetone and ethyl alcohol. There are good reasons to believe that these prices are at about the lowest possible level and that there exists great probability of a future rise in all of them. Even at these low prices, a gallon of sugar factory final molasses would yield a gross value of 18.5 cents when used in the production of these solvents. The actual total cost of production should not exceed 10 cents per gallon of molasses worked up, including all expenses. These expenses may be lowered considerably by working the process as an adjunct to a sugar factory, where cheap fuel in the form of bagasse would be available and where no transportation charges would have to be paid on the molasses used.

Patent applied for by author.

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Part II

NORMAL BUTYRIC ACID FROM WASTE MOLASSES

Butyric Acid is a Valuable and Important Acid.—Butyric acid is one of the most valuable aliphatic acids used commercially. Its steady price offers a striking contrast with the declining values that have been prevalent in the market for chemical products during the last four or five years. The present carload price in the United States for this commodity is 80 cents per pound on the basis of 100 per cent acid.

This attractive price led us to investigate the possibility of producing this organic acid from cane factory waste molasses by microbiological methods. The success obtained in our previous investigation on the production of butanol and acetone by similar methods, encouraged us to try this new study.

Difficulties Encountered by Previous Investigators.—Although fermentation processes leading to the production of organic acids have been practiced before the beginning of civilization, the mechanism whereby these processes took place on the agents responsible for them were utterly ignored. The production of butyric acid, the agent responsible for rancid butter, remained in the situation common to all these processes until Pasteur recognized the butyric fermentation as a well defined microbiological phenomenon; describing this fermentation as an anaerobic process before the Academy of Science in Paris, 1861.

Since this date, many groups of these organisms have been found. and their products of fermentation studied by several well-known investigators, among which we may mention Kirov; Baier; Fitz; Winogradsky; Buchner and Meisemheimer. These men of science agreed in that the formation of butyric acid by fermentation when using the organisms known to them, was generally accompanied by secondary reactions, producers of a variety of other substances. For instance, Buchner and Meisenheimer, when working with the "Bacillus Butyricus" Fitz, found the following products as typical of the fermentation of 100 grams glucose:—0.7 grams butanol; 2.8 grams ethanol; 1.6 grams hydrogen; 3.4 grams formic acid; 10 grams lactic acid; 7.5 grams acetic acid; and 26 grams butyric acid.

Recently, the chemists H. T. Herrick and O. E. May of the Department of Agriculture, Washington, D. C., published a circular on the production of organic acids by fermentation in which they opined that the butyric fermentation has not been applied industrially in commercial magnitude due to the great variety of substances, other than butyric acid produced during the fermentation.

The patent literature describes some processes which give the impression that the work has been done in decidedly empirical form. In some cases it is really difficult to understand how a patent could be secured on such vague, indefinite and entirely unscientific data.

Attacking the Problem.—Having acquired from the literature a knowledge of the butyric fermentation, whose synopsis is given above, we resolved to attack the problem of butyric acid production from waste or "final" sugar factory molasses, using a native bacillus.

The Bacillus is Found.—At the time of our determination to work on the butyric fermentation, we were preparing a series of extracts from the Annatto Seed (Bixa Orellana) that were to be sent to the Chicago Fair. A few seeds left over the week end in a test tube with distilled water to which a little sodium carbonate had been added, were found on next Monday morning in a very active state of fermentation. On further examination it was found that the fermenting liquid had turned from an alkaline to a decided acid reaction. Moreover, a strong butyric acid scent could be noticed. We had found the organism needed for our intended work on waste-molasses.

Our Preliminary Work.—We had learned from the experience of previous workers in this field, that the problem of the commercial production of butyric acid by microbiological methods would be practically solved with the discovery of a fermenting organism capable of producing the desired fermentation free of the secondary products obtained when working with organisms already known.

Hence, our greatest interest, once the bacillus was found, was to find out whether or not the organism would satisfy the requirements as to yield and purity of the main product of fermentation. So, as soon as we had enough quantity of the product, an analysis was made to determine its degrees of purity. The results were so highly satisfactory that a duplicate sample was sent to the Bureau of Chemistry of the Department of Agriculture, Washington, D. C. The sample was sent in the form of the barium salt of the acid. The report received from that Department stated that the product was practically entirely butyrate of barium, and that the free acid showed a high degree of purity.

These results were, of course, sufficient to give a great technical and commercial interest to our bacillus; for we had learned that the inhibiting factor militating against their commercial application, was the heterogenous product of fermentation found when working with other butyric ferments.

Other Pertinent Facts Found to Date.—As stated in our introduction this investigation is merely starting, so no definite data is available as yet. But from what has been done to date, the following additional facts may be stated:

1. The organism is exceptionally vigorous, and has shown itself capable of competing with other organisms that may gain access to the fermenting liquid. This may indicate that an absolutely pure culture is not indispensable for the growth, development, and acid production of the organism. From a commercial standpoint this is

an important factor favoring the organism; for the difficulties encountered in plant work in the prevention of contaminations in pure cultures are only too well known.

2. The organism is facultative anaerobic. This is another point of great technical importance, for strict anaerobes bring great difficulties of a technical nature, besides complications of equipment when an effort is made towards their commercial exploitation.

3. We have not determined as yet the optimum conditions of fermentation, nor the maximum obtainable yields of acid; but in the preliminary tests effected up to this time, the yields of acid have varied between 30 and 40 per cent in round numbers. These yields have been calculated on the weight of total sugars contained in the fermenting mass.

4. Accepting an average value between the two figures given above, as the probable commercially obtainable yield, we would have that a gallon of "final" or waste molasses, would yield about 2.1 pounds of the acid with a gross value of $1.68.

Finale.—As closing words I wish to express my firm conviction in the glorious future awaiting fermentation processes in the field of modern industrial chemistry, and especially in their application to carbohydrate materials. Indeed, many of our more important organic chemical products will be manufactured in a not remote future by methods of industrial microbiology.

The beautiful islands of the Caribbean offer unexplored treasures in their microbiological flora, and are destined to become great chemical laboratories of the future where fermentation industries will be predominant; and the future of our great sugar industry will be most intimately connected with such methods of manufacture.

Patent will be applied for by the author.

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