AN UNKNOWN FACTOR STIMULATING THE FORMATION OF BUTYL ALCOHOL BY CERTAIN BUTYRIC ACID BACTERIA[1]

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The question of growth stimulants for microörganisms has long been one of the most interesting and widely discussed subjects of biological research. The widespread occurrence of growth-stimulating factors has been well demonstrated, and a wide variety of microörganisms have been shown to be affected by such factors. Peskett (1933) has recently given a general review of the subject but certain aspects dealing with the occurrence of stimulants in plant materials should be emphasized. Growth-factors have been found in lemon juice, carrots, potatoes, spinach, radishes and many other plant tissues. The organisms affected by some of these factors include yeasts (Robertson and Davis (1923)) and bacteria. Among the latter are butyric acid bacteria (Ruschmann and Harder (1931)), hemolytic organisms (Morgan and Avery (1923)), staphylococci (Leichtentritt and Zielaskowski (1922)), streptococci (Thompson (1929)), the pneumococci (Thjötta and Avery (1921), Kollath (1926), Kopp, (1927)), and the tubercle organisms (Uyei (1930)). Little is known about the rôle played by the stimulating factors or about their actual chemical nature. Thompson (1929) suggests that the growth-promoting action of potato extract is due to its nitrogenous food constituents rather than to an "accessory growth factor." Uyei (1930) on the other hand, investigating the stimulating action of potato on the tubercle bacillus, found that a protein preparation of potato

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had no growth-promoting activity. He also found that the known carbohydrate constituents of the potato had slight stimulating effects which could not, however, be compared with that brought about by the extract itself.

In a previous paper (McCoy, Fred, Peterson and Hastings (1930)), it was reported that many butyric acid bacteria grew well and formed solvents (butyl, alcohol, etc.) on potato mash, but did not give such results on corn mash and other substrates. The present paper deals with the unknown factor which is responsible for this difference in fermentation and with the general distribution in nature of this factor.

EXPERIMENTAL

Cultures and media. Pure cultures of the anaerobic butyric-acid-forming bacteria were used, and according to bacteriological tests were free of contaminating forms. The detailed description of these organisms has not been completed and for the present the bacteria are listed by number. While all of these cultures are butyric-acid-producing forms, they have not been typed within the group and therefore some of them may be duplicates. Cultures 19, 21, 22 and 25 as listed in a former paper (McCoy, Fred, Peterson and Hastings (1930)) and in addition, cultures 36, 37, 38 and 39 were employed.

The media used were as a rule mashes containing 4 per cent of the grain or tuber calculated on the dry basis. Speakman's (1923) peptone-inorganic salt medium was used in a few experiments with 4 per cent glucose or purified starch as carbon sources. Throughout the early work 200 cc. of the medium in 250-cc. Erlenmeyer flasks were used. Later 8-inch test tubes (1 inch diameter) containing 35 cc. of medium were employed. Inoculation was made from a twenty-four hour culture of the organisms in corn mash. One per cent of inoculum was used in every case, and the cultures were incubated at 37°C.

The degree of stimulation was evidenced by turbidity of the supernatant liquid, by rapidity of gas evolution, by "head" formation, and by the characteristic butyl-alcohol odor. Throughout the investigation the conclusions arrived at by means of these

observations were checked by chemical analysis for butyl alcohol, ethyl alcohol and acetone.

Analytical methods. Ethyl and butyl alcohols were determined by Johnson's (1932) micro-method. Acetone was determined iodometrically.

Products of butyric acid organisms from corn and from potato. The products from these two materials are given in table 1. Corn is seen to be a poor substrate, very little (10 per cent) of the starch being fermented. The products were mainly acetic and butyric

TABLE 1

Products from fermentation of corn and potato

(Calculated for 100 grams dry matter)

CULTURE	MEDIUM	BUTYL ALCOHOL	ETHYL ALCOHOL	ACETONE ALCOHOL	TOTAL SOLVENTS
		grams	*grams*	*grams*	*grams*
21	Corn	1.01	0.09	1.04	2.14
21	Potato	13.60	0.49	0.97	15.06
36	Corn	0.18	0.89	0.82	1.89
36	Potato	12.97	0.62	1.16	14.75
22	Corn	0.46	1.06	1.28	2.70
22	Potato	12.68	1.32	0.92	14.92
25	Corn	0.23	1.01	0.97	2.21
25	Potato	1.55	0.91	1.16	3.52
19	Corn	0.19	0.87	0.65	1.71
19	Potato	1.21	0.98	1.08	3.27
38	Potato	2.90	1.12	1.16	5.15
37	Potato	2.81	1.27	1.01	5.09
39	Potato	1.41	1.15	1.04	3.60

acids and acetone, with very small yields of alcohols. All the organisms used showed a decided increase in butyl alcohol production from potato. This increase was correlated with an increased fermentation of starch, amounting in certain cases (cultures 21, 22 and 36) to 80 per cent. It is noteworthy that in all cases most of the increase in solvents was accounted for by butyl alcohol. The production of this substance was therefore taken as the criterion of stimulation in further work. Besides the five cultures tested on both corn and potato, three other cultures were tested on potato alone. None of these produced appreciable amounts

of butyl alcohol. The same lot of potatoes was used in all these fermentations so that the difference in butyl alcohol production can be explained only on the basis of strain differences. Although three of the cultures showed about the same response to potato, culture 21 was used for most of the later experiments because somewhat more data were available for it.

During the course of the investigation several varieties of white potatoes were fermented with culture 21. Three of the varieties were fermented vigorously, while two gave no better fermentations than corn mash. All samples of Wisconsin potatoes that have been tried brought about vigorous fermentations. These potatoes have, therefore, been used in the preparation of potato extracts for further work.

Fermentation of other starchy materials. Several other starch-containing natural products were fermented by culture 21 to determine whether the white potato was the only source of the stimulant. Only one of these materials, the thick-skinned sweet potato or yam showed any stimulative effect. It is curious that Jersey sweet potatoes, which are so similar to the yam showed no effect whatever. Wheat germ gave slightly higher yields of butyl alcohol than corn, but rice, oats and barley did not.

Effect of various supplements on the fermentation of corn by culture 21. It was thought that perhaps the failure of culture 21 to bring about a good fermentation of corn mash might be due to its inability to utilize the protein and starch of corn. Various modifications of the corn medium were therefore made in an attempt to improve the solvent production. The results are given in table 2. The addition of peptone (1 per cent) increased the butyl alcohol production to some extent, but higher concentrations added (3 per cent) had no further affect. An increased sugar concentration also increased the solvent production in corn mash, as is shown by the higher yield from corn which had been digested with malt diastase. Such an increase in sugar content also explains the slight but definitely increased yield of butyl alcohol from sprouted corn, since the diastatic enzymes of the corn germ attack the starch during germination. In none of these fermentations, however, was the yield of butyl alcohol compar-

able to that obtained from potato. Furthermore, the addition of a water extract of potato, either crude or purified, enabled the organism to ferment corn as vigorously as it did potato.

Preparation and purification of potato extract. Since the active principle in potato was found in a water extract, large quantities of crude extract were prepared for future use by the following procedure. The washed raw potatoes were ground, the juice was pressed out, the residue was washed twice with distilled water and pressed. The juice and washings were united, the starch was allowed to settle, and the clear supernatant liquid was siphoned off

TABLE 2

Effect of various supplements on production of solvents from corn by culture 21

(Calculated for 100 grams dry matter)

ADDITIONS TO 100 CC. 4 PER CENT CORN MASH	BUTYL ALCOHOL	ETHYL ALCOHOL	ACETONE	TOTAL SOLVENTS
	grams	*grams*	*grams*	*grams*
None	0.14	0.97	0.98	2.09
1 gram peptone	3.18	0.89	1.74	5.81
2 grams diastase (no digestion)	0.23	1.28	0.72	2.23
2 grams diastase (digested)	4.83	0.12	0.65	5.60
Sprouted corn used	0.86	0.99	1.73	3.48
21 cc. crude potato extract*	14.30	0.60	1.09	16.99
6 cc. purified potato extract*	9.60	0.43	0.96	10.99
15 cc. purified potato extract*	11.70	0.17	1.18	13.05
30 cc. purified potato extract*	14.42	0.22	0.95	15.59

* One cubic centimeter of extract represents 1.0 gram raw potato.

and filtered. The solution was then heated to precipitate heat-coagulable proteins, and these were filtered off. The filtrate was sterilized in flasks and stored until needed. Since this crude extract was found to contain starch, sugar and protein, it was purified by precipitating these substances. An excess of ammonium hydroxide and about 30 cc. of a saturated solution of lead acetate were added to each 100 cc. of crude extract. The solution was filtered several times in a Büchner funnel through Norit. The excess ammonia was then boiled off, and the lead removed with H_2S. This procedure was found to remove all detectable traces of starch (iodine test), sugar and substances hydrolyzable to sugar

(Fehling's test), and proteins (biuret test). As may be seen from table 2, the extract is practically as stimulating after this treatment as before.

Effect of other vegetable extracts on the fermentation of corn. The occurrence of the stimulant in plant materials other than potato was determined by testing extracts from such materials prepared in the same way as the potato extract. Dry materials, such as peas and beans, were first soaked in water, pressed and treated in the usual way. Concentrated corn-steep which was obtained from the Corn Products Refining Company was also purified by the lead acetate, etc., treatment. The extracts were tested for their stimulatory action on the fermentation of corn by culture 21. The curves in figure 1 show the stimulative effect of some of these extracts as measured by butyl alcohol production. All the extracts were found to contain the stimulant, but in varying concentrations. For example, a degree of stimulation which required 9 cc. of malt-sprout extract could be brought about by 1.5 cc. of pea or potato extract. Corn-steep is one of the materials richest in the stimulant. This may seem contradictory in view of the failure of corn mash alone to undergo a butyl fermentation. This apparent anomaly may be explained by the concentration of the factor in the preparation of the corn-steep. In corn meal, while probably not absent, the factor is present in too small a quantity to bring about any considerable fermentation of starch. It should be noticed that all the curves tend to flatten out after the maximum butyl-alcohol production is reached, which maximum is nearly the same in all cases. This shows that the effect is proportional to the concentration of the stimulant up to a certain point, but that thereafter, a higher concentration has no effect.

Certain other plant materials contained the stimulant to a more marked degree than those shown in figure 1. The curves representing the effects of extracts of cabbage, orange and lettuce are shown in figure 2. The much greater concentration of the stimulant in these materials (on the dry weight basis) made it necessary to modify the horizontal scale on the figure. The general shape of the curves is very similar to those in figure 1. In the case of the lettuce extract the concentration of ammonium acetate due to the

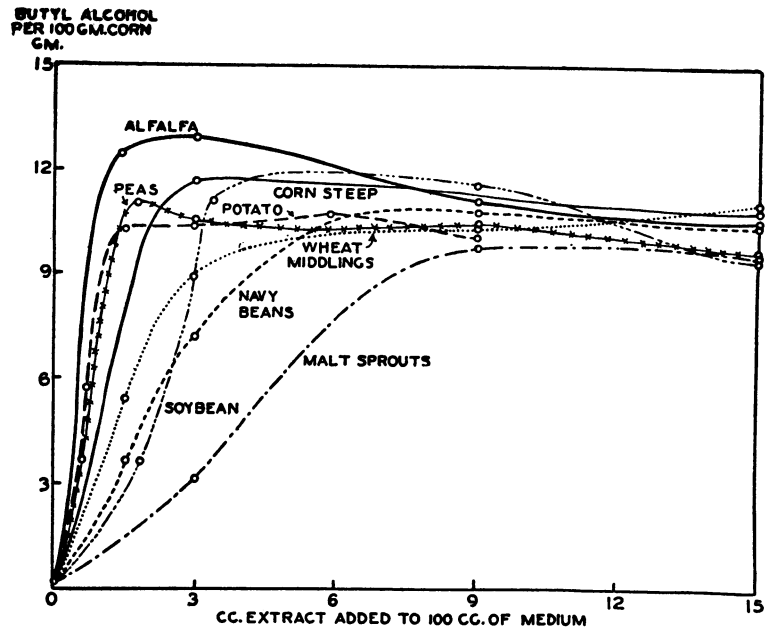

FIG. 1. EFFECT OF PLANT EXTRACTS ON FERMENTATION OF CORN BY CULTURE 21
(Excepting corn-steep 1 cc. is equivalent to 1 gram dry matter)

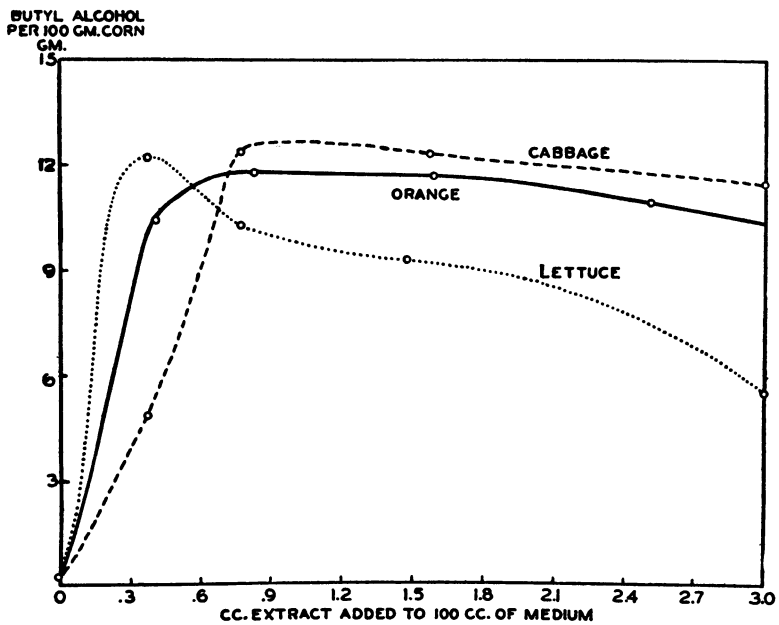

FIG. 2. EFFECT OF CABBAGE, ORANGE AND LETTUCE EXTRACTS ON FERMENTATION OF CORN BY CULTURE 21
(One cubic centimeter is equivalent to 1 gram dry matter)

method of preparation was so large that it became toxic in the larger amounts added. This explains the dropping off in the curve. Lettuce, cabbage and orange seem to contain from five to ten times as much stimulant per unit dry weight as the other sub-

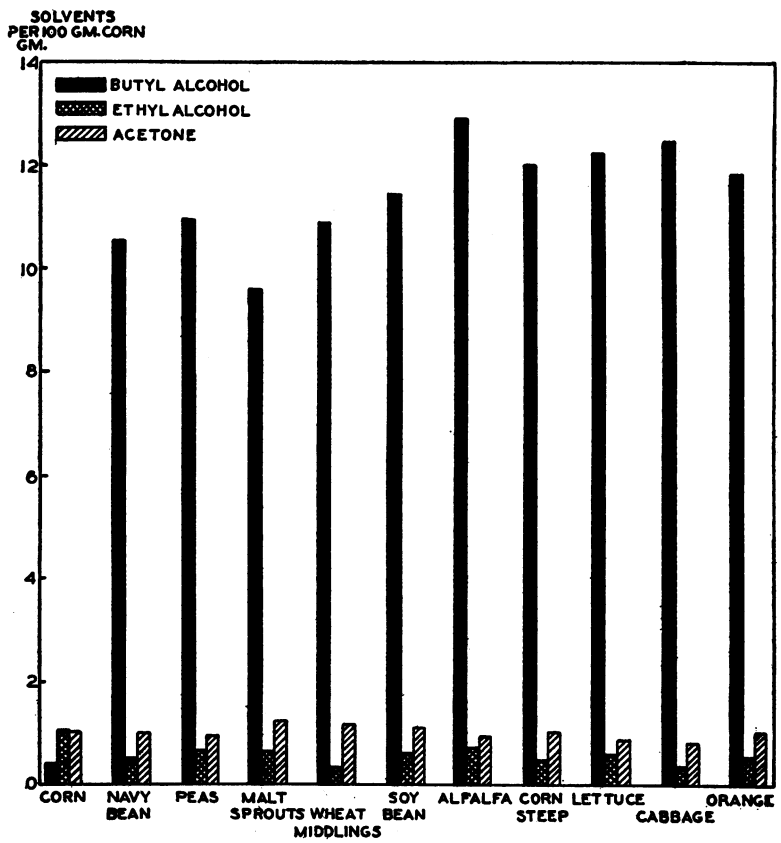

FIG. 3. EFFECT OF PLANT EXTRACTS ON PRODUCTS FORMED FROM CORN BY CULTURE 21

stances tried. An idea of the relative concentration of stimulant in these substances may be obtained from the slope of the curves from the control point to the flat portion. Lettuce seems to be the most concentrated source of the stimulant, with orange and cabbage nearly as concentrated. Alfalfa seems to be the best of

the other substances, and malt sprouts apparently is the poorest. The effect of the various plant extracts on the yield of all solvents by culture 21 is given in figure 3. The data are given only for the point of maximum stimulation. As was indicated previously (table 1) the only one of the three solvents to show any appreciable increase was butyl alcohol.

In order to determine whether these extracts stimulated other butyric acid bacteria besides culture 21, soy-bean, pea and corn-

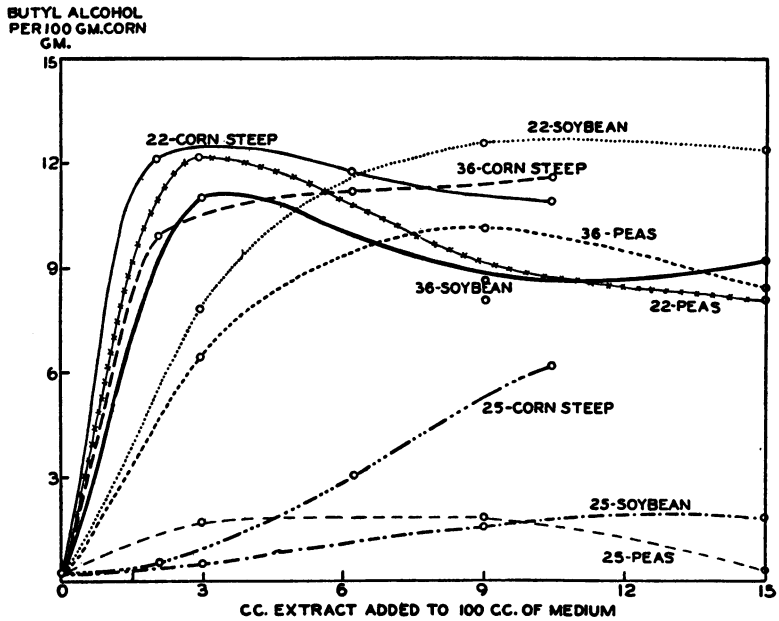

FIG. 4. EFFECT OF PLANT EXTRACTS ON FERMENTATION OF CORN BY CULTURES 22, 25 AND 36

(Excepting corn-steep 1 cc. is equivalent to 1 gram dry matter)

steep extract were added to corn and fermented with several other strains of butyric acid bacteria. The curves in figure 4 show the effect of the extracts on these organisms. Culture 25 was very slightly affected by the stimulant, while cultures 22 and 36 showed a response quite similar to that of culture 21. Attention is called to the fact that these organisms exhibited the same differences in fermentation when grown on potato (table 1). Figure 5 summa-

rizes the date on the solvent production of these organisms at the points of maximum yields.

It should be noted that the solvent production by the three strains showing marked stimulation, (i.e. cultures 21, 22 and

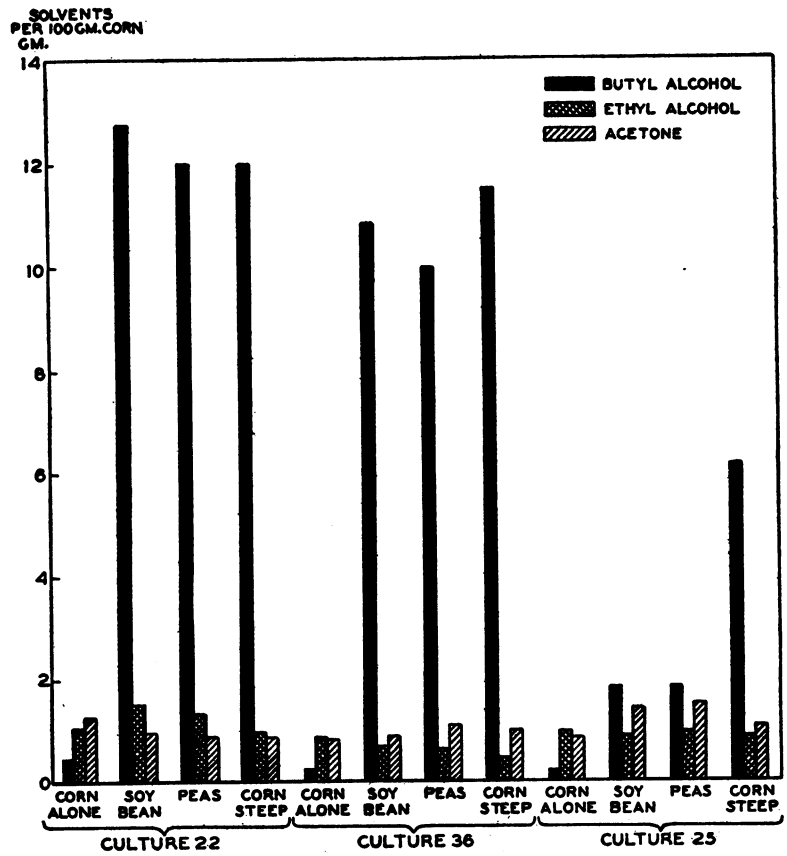

FIG. 5. EFFECT OF PLANT EXTRACTS ON PRODUCTS FORMED FROM CORN BY CULTURES 22, 25 AND 36

36) is of about the same magnitude, and that this same yield is obtained irrespective of the source of the stimulant (figs. 3 and 5). It seems logical to assume that the same factor is concerned in all cases, since it is improbable that there would be more than one factor which would resist the treatment involved in the prepara-

tion of the extracts (heating, filtration through Norit, treatment with ammoniacal lead acetate, and precipitation of lead sulfide) and which would at the same time affect different butyric acid bacteria in an identical manner.

SUMMARY

An unknown substance which greatly stimulates the fermentation of corn-mash by certain butyric acid bacteria has been found in potatoes, yams, oranges, lettuce, cabbage, alfalfa, soy- and navy beans, wheat middlings and malt sprouts. This substance appears to be low or lacking in corn, rice, oats and barley.

The effect of the unknown stimulant is greatly to increase the destruction of starch and to increase the production of butyl alcohol more than tenfold. Yields of other solvents (acetone and ethyl alcohol) are not affected.

While many plant materials contain the stimulant, the concentration varies. On the basis of dry matter, lettuce, cabbage and orange contain from five to ten times as much stimulant as the other plant materials tested.

Different strains of the butyric acid bacteria have been shown to differ widely in the degree of response to the stimulant.

A method of preparing extracts of vegetable materials involving purification with ammoniacal lead acetate is described. Such extracts are free from detectable traces of glucose, carbohydrates hydrolyzable to glucose, and proteins.

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