THE MANNITOL-PRODUCING ORGANISMS IN SILAGE

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INTRODUCTION

The chemistry section of the Iowa agricultural experiment station has shown (Dox and Plaisance, 1917 a and b) that mannitol is a normal constituent of silage and has reported experiments indicating that "it is formed in silage fermentation by bacterial reduction of the fructose-half of the sucrose molecule." In silage, the mannitol is produced in considerable amounts, simultaneously with the acids, the carbon dioxide and the alcohol and "its presence accounts in large measure for the deficit noted when the sum of these products is balanced with the fermented sugar."

The results reported in the present paper¹ deal with the isolation from silage of organisms capable of producing mannitol when grown in pure cultures in corn, corn juice, and various other materials.

HISTORICAL

The presence of mannitol in the higher plants, in both the higher and lower fungi, and in various fermented materials such as wine, vinegar and sauerkraut, as well as its production by organisms, has already been dealt with in some little detail in the publications of the Iowa station. It is evident that mannitol fermentation has long been known and that it is more or less common.

¹ The work herein dealt with was carried out in 1917 and was to have been reported at the meeting of the American Society of Bacteriologists in that year. The National Research Council, however, requested that the report be delayed because of the possible use of mannitol in the manufacture of explosives.

METHODS USED

In order to prevent repetition, some of the materials used are here described. The corn juice was secured by pressing green corn, while the stover juice was obtained by soaking corn stover in water for twelve hours and then pressing. The corn silos were made by packing chopped green corn, and the stover silos by packing chopped stover, and adding a calculated amount of water and usually about 5 per cent sucrose, calculated on a dry basis. For most of the silos, the material was packed in quart Mason jars but in a few instances bottles or flasks holding from 1 to 2 liters were used.

Corn juice agar was made by adding 1.5 per cent agar and 1 per cent pepton to the corn juice while the stover juice agar was made by adding 1.5 per cent agar, 1 per cent pepton and 5 per cent sucrose to the stover juice; the stover juice agar was commonly cleared with an egg when it was wanted for plating but this was not necessary with the corn juice agar. The corn juice agar was more satisfactory than the stover juice agar because it was lighter in color and apparently gave a more satisfactory growth.

The method of isolating and determining mannitol in silage was that used in the former work at the Iowa station which has already been referred to. Mannitol was determined in liquid cultures by evaporating 100 cc. aliquot to dryness on a steam box; the residue was then extracted five times with boiling 95 per cent alcohol (about 15 cc. of alcohol in each portion) and the combined extracts filtered as soon as cold. After standing over night the mannitol had crystallized; the crystals were sucked dry, recrystallized from water and alcohol, dried and then weighed.

RESULTS SECURED

The rôle of microorganisms in mannitol production

Although in the previous work at the Iowa station the production of mannitol was secured by inoculating sterilized stover (plus sucrose and water) with a decoction of a leaf of corn silage

and was not secured in "antiseptic" silage made by adding ether to corn, it seemed desirable to repeat and extend these experiments in order to confirm the relationship of organisms to mannitol production. The inoculation of sterilized corn or stover silos with a bit of normal silage gave mannitol production regularly. The lack of mannitol in corn silos treated with various chemicals and held at room temperature for periods that gave mannitol with the untreated corn is shown in table 1.

When silage from the silos to which the various chemicals had been added was examined under the microscope a very few lightly stained bacteria were the only microorganisms observed and these, in all probability, represented organisms that were

TABLE 1

The influence of various chemicals on mannitol production, room temperature incubation

ADDED TO THE CORN	PERIOD OF HOLDING	MANNITOL
	<i>days</i>	
Nothing added, normal fermentation.....	14	Present
Ether.....	14	None
Chloroform.....	14	None
Chloroform and toluol.....	14	None
Formaldehyde.....	14	None

present on the corn at the time the silos were filled. The normal silage on the other hand showed very large numbers of well stained bacterial and yeast cells and thus presented a very different picture than the treated silage. When these statements are compared with table 1 it is evident that when microorganisms developed normally mannitol was produced, while when the growth of microorganisms was prevented by various chemicals no mannitol was formed.

The isolation of mannitol-producing organisms

The isolation of organisms capable of producing mannitol was attempted by plating out samples of silage on corn juice or stover juice agar. Representative organisms developing on the plates were grown on agar slopes (usually the same agar as

that used for plating) and were then tested for mannitol-producing power by inoculating into sterile corn juice, sterile stover juice, a sterile corn silo or a sterile stover silo, allowing growth to go on for a period varying from a few days to several weeks, and then examining the material for mannitol.

Attempts were made to isolate mannitol-producing organisms from a number of samples of silage that had been ensiled several months but only negative results were secured. Yeast² colonies were commonly present on the plates in considerable numbers and often made up the greater part of the developing flora; many of these were tried out for mannitol-producing power but when the yeasts were in pure culture mannitol was never found. Other types of silage were then studied and the first mannitol-producing organism isolated —M39— was secured from silage fourteen days old that was made by ensiling green corn from the greenhouse; the silage contained 1.09 per cent mannitol at the time it was plated out. The colony from which M39 was secured was very small and comparatively few of its kind were present; the organism was found capable of producing mannitol in sterile corn juice, in sterile stover juice, in sterile corn silos, and in sterile stover sucrose silos and has consistently given mannitol in a large number of trials.

Mannitol-producing organisms were readily isolated from a sample of corn juice that was covered with oil (to keep down mold growth) and allowed to ferment spontaneously and that showed, after a short period, considerable quantities of mannitol. A direct microscopic examination showed many yeast cells and still larger numbers of bacteria. On plating out, on corn juice agar, material from both the upper and lower layers of this fermenting juice, colonies similar to those of M39 were found in large numbers, together with many yeast colonies. When the colonies similar to those of M39 were streaked on agar slopes and then inoculated into either sterile corn juice or a sterile silo, mannitol was found in considerable amounts after the usual holding period.

² The term yeast is used to indicate organisms reproducing by budding.

Evidence that organisms of the type of M39 are concerned in the production of mannitol was furnished unexpectedly in two instances as follows:

1. A control stover silo, which had been opened after sterilization only for the purpose of adding sterile water, contained considerable mannitol when it was examined after a period of about two weeks. Microscopic examination showed many organisms morphologically resembling M39 and corn juice agar plates inoculated with the material yielded colonies like those of M39; transfers were made to corn juice agar and when the organisms were inoculated back into the usual test materials mannitol was found in considerable quantities after a holding period of from ten to twenty days. It seems that the presence of the mannitol-producing organisms in the silo was due to accidental inoculation since the organism is not exceptionally heat resistant and accordingly would not be expected to survive the heating during sterilization; moreover the other control showed neither organisms nor mannitol.

2. One of the yeasts isolated from silage was inoculated into a sterile stover silo and after the usual holding period an examination showed the presence of mannitol. When the silage was examined microscopically, in addition to the yeast, an organism morphologically similar to M39 was found in large numbers; this organism was isolated by plating on corn juice agar and proved capable of producing mannitol when inoculated into the usual test materials. Although a microscopic examination of the original yeast culture had shown no bacteria, it is entirely possible that a very small number of organisms of the M39 type may have been present in the culture and had thus been inoculated into the silo along with the yeast. Contamination of the silo, either at the time of inoculation or later when the silo was opened to release pressure, is another means of explaining the results secured.

Influence of oil at the surface of the liquid on mannitol production

Since the formation of mannitol is to be looked upon as a reducing process, it was thought that the addition of sterilized

oil to the flasks of liquids to be fermented might materially modify the results obtained. A number of comparisons of oiled and unoiled material were made at room temperature, using a juice expressed from cane and to which 2 per cent sucrose had been added before sterilization; the results secured are presented in table 2. From the data given it is evident that under the conditions employed, a larger percentage of mannitol was secured with oil than without it. Because of the gas liberated, which probably drives off much of the unconsumed oxygen, the conditions in the unoiled flasks must be, to a certain extent, anaerobic and this, in all probability, explains the formation of mannitol in the absence of oil. Whether the presence of air results in a smaller production of mannitol or in a destruction

TABLE 2

The influence of oil on mannitol production, room temperature incubation

ORGANISM	PERIOD OF INCUBATION	PER CENT OF MANNITOL	
		Without oil	With oil
	<i>days</i>		
M39	12	1.58	1.75
M363	12	0.60	0.83
M308	12	Trace	1.79
M439	12	0.44	1.60
M283	12	0.46	0.71

of a portion of that produced is impossible to determine from the data available.

In general, during the fermentation of the juices used there was a pronounced change in color; in the unoiled flasks the lighter portion involved mainly the lower depths, due presumably to the air above, while in the oiled flasks practically the entire liquid was involved. It seems probable that the change in color involved a reduction of some indicator present in the fermenting juice, the process being essentially similar to the reduction of litmus by many organisms, although it is possible that the change in color was due to the production of acid; the latter explanation is less acceptable than the former since the change in color in general agrees with the state of anaerobiosis.

Materials yielding mannitol

The organisms that were found capable of producing mannitol in the usual test preparations were studied as to their ability to produce it in a number of other materials. Two cultures from different samples of silage were inoculated into sterilized carrot juice³ but no mannitol was secured. Both table and sugar beet juices were tried, two cultures on the former and four on the latter, but with negative results. Cabbage juice gave considerable quantities of mannitol with each of the three organisms inoculated into it and in general there was abundant gas formation. With the juices of the carrot, beet and cabbage there was a change in color similar to that secured with the corn and stover juices and in all cases the turbidity which developed indicated that the organisms were growing well. The failure to secure the production of appreciable amounts of mannitol with carrot and beet juice was undoubtedly due to the lack of the proper carbohydrate materials in them. A considerable number of flasks of apple juice were sterilized and inoculated with different cultures but there was no evidence of growth in any of them and the few flasks examined showed no mannitol; the same results were secured when the apple juice was neutralized before sterilization. Considerable quantities of mannitol were secured when sunflower stalks, leaves and blossoms were chopped, packed in jars, sterilized and inoculated with pure cultures of different mannitol-producing organisms, or when the sunflower material was packed in jars and allowed to ferment spontaneously. Cane yielded considerable quantities of mannitol when treated similarly to the sunflowers, as did also mixtures of stems, leaves and blossoms of dandelions.

One lot of stover juice (juice x) was found to contain only very small amounts of hexose sugars and accordingly it was used as a basis for testing out various materials. When 5 per cent cane sugar was added to this juice and the material sterilized, tests showed no appreciable inversion of the sugar; on inoculation with some of the mannitol-producing organisms

³ The various juices were secured with a powerful press.

only traces of mannitol were found and it seems probable that this came from the small amounts of hexose sugars present in the juice. In the light of these tests it seems difficult to explain the value of the cane sugar in the stover juice, stover silos, etc.; it is entirely possible, however, that inversion might have occurred in some cases, even if not with juice x. By the addition of 5 per cent invert sugar to juice x, sterilization, and the inoculation of mannitol-producing organisms considerable quantities of mannitol were secured; growth was apparently very rapid and there was usually a pronounced change in color and the evolution of considerable gas. When honey (usually 8 per cent was used) was added to juice x, there was

TABLE 3
Mannitol in silage made from stover plus various substances, room temperature incubation

MATERIAL ADDED TO STOVER	MANNITOL
	<i>per cent</i>
Glycerol.....	0
Galactose.....	0
Glucose.....	0
Fructose.....	3.71
Maltose.....	0
Lactose.....	0
Inulin.....	0.40
Starch.....	0

an exceptionally heavy gas production, a pronounced change in color and the formation of considerable quantities of mannitol.

A series of silos was made by cutting up corn stover (containing practically no sugars) adding various substances, packing in Mason jars and then sterilizing, after which organism M39 was inoculated. The results of mannitol determinations made on the silage after a suitable holding period at room temperature are shown in table 3. From the data presented it is evident that fructose and inulin yielded mannitol while glycerol, galactose, glucose, maltose, lactose and starch did not. Many lots of silage made by inoculating various organisms into sterilized stover plus sucrose have, as already stated, yielded mannitol.

It seems then that only fructose, or materials giving fructose on hydrolysis were capable of yielding mannitol when acted on by the mannitol-producing organisms studied. The small amount of mannitol produced in the inulin stover silo, as well as in many of the sucrose stover silos, was undoubtedly due to the inability of the organisms to hydrolyze these materials and to the small amount of hydrolysis which occurred during the process of sterilization. The variations in the amounts of mannitol produced in the sucrose stover silos were very likely due to differences in the amount of hydrolysis, and this was materially influenced by the amount of acid present and by the extent of the heating. Gayon and Dubourg (1894; 1901) found that only fructose or its moiety yielded mannitol and Brown has shown how the configuration of fructose is such that it alone can be changed to mannitol by organisms.

It is entirely possible that certain organisms may be able to produce mannitol from such materials as sucrose and some results have been secured which indicate that one of the cultures isolated is able to do this. It seems quite certain, however, from results secured with the use of bouillon to which sucrose was added, that most of the cultures isolated are unable to change sucrose to mannitol.

Distribution of the mannitol-producing organisms

Since mannitol is a normal silage constituent and is produced by the action of microorganisms, it would be expected that mannitol-producing organisms would be rather widely distributed about barns where silage is used, due to the scattering of silage and of manure from animals consuming silage. A number of materials have been tested for mannitol-producing organisms by inoculating them into sterile corn juice, flooding the juice with sterile oil in order to keep down the growth of molds and then determining the presence or absence of mannitol after a suitable incubation period at room temperature. The production of mannitol has been secured with soil from a farm yard and also with milk, but the trials made are too few in number to allow of any conclusions regarding the extent of the contamination of these materials.

The per cent of mannitol produced in various materials

The per cent of mannitol produced in different materials varied widely. While this was due to a large extent to variations in the per cent of total sugar, as well as to variations in the make-up of the sugar in the original materials, differences in the efficiency of the different organisms tried undoubtedly played a very large part. Table 4 presents data, other than those already presented, showing the per cent of mannitol produced under different conditions and in various materials. Many other determinations were made only to find out whether mannitol was present in traces or in considerable quantities and the results are of course omitted.

TABLE 4

The per cent of mannitol produced under different conditions, room temperature incubation

MATERIAL	INOCULATION	PERIOD OF HOLDING	MANNITOL
		days	per cent
Cane juice plus 2 per cent sucrose.....	M283 plus yeast	12	1.85
Cane juice plus 2 per cent sucrose.....	M283 plus yeast	12	1.05
Cane juice plus 2 per cent sucrose.....	M308 plus yeast	12	1.65
Cane juice plus 2 per cent sucrose.....	M308 plus yeast	12	1.00
Cane juice plus 2 per cent sucrose.....	M439	12	0.49
Cane juice plus 2 per cent sucrose.....	M308	12	0.52
Green corn silage.....	M39	18	0.59
Corn juice.....	M393	12	0.60
Corn juice.....	M393	20	0.92

The destruction of mannitol

The data already reported by the Iowa station show that, in a silo, part of the mannitol produced is destroyed. Table 5 shows the per cent of mannitol present at various times in stover silage containing sucrose inoculated with organism M39 and held at room temperature. A series of silos were prepared and a different one used for each determination.

From table 5 it seems that, at least with the organism used, the production of mannitol was accompanied or followed by its partial destruction.

Organisms having mannitol-producing powers

A considerable number of organisms capable of producing mannitol were isolated from various samples of silage and studied morphologically, culturally, and biochemically. The results showed that the organisms cannot be considered to be of one type. Most of the cultures isolated produced no appreciable change in milk and undoubtedly should be classed as *Bacillus manniticus* of Gayon and Dubourg, but one of the cultures in particular produced a coagulation in litmus milk with an extensive reduction of the litmus and its general characteristics indicated that it should be classed as *Bact. casei*. Certain of the rod-shaped (Kruse 1910; Orla-Jensen 1919) lactic acid

TABLE 5

The per cent of mannitol at various times in stover silage containing sucrose and inoculated with organism M39, room temperature incubation

PERIOD OF HOLDING	MANNITOL
<i>days</i>	<i>per cent</i>
6	1.41
8	2.41
10	2.63
12	1.60
14	1.54
20	1.38

organisms have been shown by a number of investigators to produce mannitol. With this group of organisms, however, mannitol production is not a general characteristic since a considerable number of cultures from sources such as milk, silage and cow feces were examined for mannitol production by inoculating into satisfactory media but only with negative results.

From the findings reported it seems that the production of mannitol in silage is not the result of the action of organisms present in silage alone but is brought about by the activity of organisms that have been shown to produce mannitol in other materials such as wines, etc. The conditions, such as a lack of oxygen and the presence of sugar, existing in silage during the period of active fermentation are undoubtedly very favorable

to the type of change resulting in the formation of mannitol from fructose. The rod-shaped lactic acid organisms constitute a group that is present in silage in enormous numbers (Hunter and Bushnell, 1916; Sherman, 1916) and while many of these do not produce mannitol it seems probable that mannitol-producing forms may be expected among them.

CONCLUSIONS

1. The production of mannitol in silage is very evidently due to bacterial action.

2. Mannitol-producing organisms were readily isolated from silage, provided it had been ensiled recently. They were also secured from a sample of fermenting corn juice.

3. In liquids, more mannitol was produced when they were flooded with oil than when they were not.

4. Mannitol was produced, by the organisms isolated, in the juice of cabbage and in silage made from corn, sunflowers, cane or dandelions, but not in the juice of carrots, beets, or apples. Fructose, or materials giving fructose on hydrolysis, such as sucrose or inulin, also yielded mannitol when added to stover before sterilization; it is probable that the hydrolysis was due to the heating and the acid present and cannot be accomplished by the organisms although there may be variations among the organisms in this respect. Glycerol, galactose, glucose, maltose, lactose and starch did not yield mannitol when added to stover before sterilization while honey gave large amounts.

5. The mannitol-producing organisms were found to be present in soil from a farm yard and in milk.

6. The per cent of mannitol produced in different materials varied widely, due undoubtedly to a large extent to variations in the types and amounts of sugar present.

7. With the only organism that was tried, the production of mannitol was accompanied or followed by its partial destruction.

8. The organisms producing the mannitol in silage cannot be considered to be of one type.

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