Studies on the Formation of Acrolein in Distillery Mashes[1]

DORIS E. MILLS, W. D. BAUGH AND H. A. CONNER

Research Division, National Distillers Products Corporation, Cincinnati, Ohio

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During the distillation of alcohol from a grain mash, it may be noted on relatively rare occasions that the product has a pungent odor or even may be mildly lachrymatory. The term "peppery" has been applied to such distillates. The whiskey is sometimes known as "red eye". It has been known for many years that this "peppery" character is due to the presence of acrolein. The same or a similar phenomenon may occur in the manufacture of brandy. In the latter case it has been shown that bacterial action during the yeast fermentation is responsible for the production of the aldehyde.

Very little has been published concerning this phenomenon as it occurs in grain mashes. This paper contains the results of a study on some of the factors influencing the microbial production of acrolein in these mashes.

Voisenet (1918) isolated an organism *Bacillus amaracrylus* (*Bacillus polymyxa*) which he considered to be responsible for the formation of acrolein during the fermentation of wines. He has reported the production of acrolein by this organism on a substrate containing glycerol, peptone and inorganic salts.

Humphreys (1924) found that all of his strains of *Clostridium welchii* (*Clostridium perfringens*) were capable of producing acrolein from a substrate containing glycerol.

Other studies on the bacterial formation of acrolein include those of Reynolds *et al.* (1939) and Michelson and Werkman (1940). These workers showed that acrolein is an intermediate in the fermentation of glycerol by *Escherichia* (*Citrobacter*) *freundii*.

Quite early in this study it was evident that the organism which is the subject of this report was distinct from those studied by previous investigators.

EXPERIMENTAL METHODS

Isolation of the organism. The bacterium on which these studies were made was isolated from a sample of rye mash or beer which had been fermented for 72 hours and which had yielded a lachrymatory distillate. Several dilutions of the beer were plated on tomato juice agar, and the plates incubated anaerobically at 30 C for 5 days. One of the organisms which appeared formed long chains in tomato juice broth. Eventually this bacterium proved to be the one which produced acrolein.

Fermentations. The substrates used in this study included grain mashes of either distillery or laboratory origin, synthetic media, tomato juice broth (essentially the substrate used by Garey *et al.* (1945) but without the agar) and a tomato juice broth containing washed grain residue and glycerol. Details are given later.

Cultures of bacteria were carried in stabs of a commercial substrate known as "L" agar (B-B-L). Transfers from these slants or stabs were made to the tomato juice broth, usually 24 hours before the inoculum was to be used. The resulting tomato juice broth culture was used to inoculate the experimental mashes.

Unless otherwise stated, the acrolein fermentations were conducted by inoculating 100-ml portions of sterilized mashes with the acrolein-producing bacterium and incubating the cultures at 30 C.

Acrolein was removed from the cultures by diluting the latter with 100 ml of water and distilling until 100 ml of distillate had been collected. The method of Circle, Stone, and Boruff (1945) was used to determine the acrolein in the distillate.

RESULTS

Attempts to transmit the phenomenon of "pepper" in series, by inoculating a fresh grain mash with the mash from which the acrolein producer had been isolated, gave completely negative results. Similarly, when pure cultures of isolates from "peppery" mashes were used to inoculate fresh mashes, no acrolein was produced.

It had been observed as a result of successive samplings of plant mashes that in many cases those which gave a "peppery" distillate at the end of 80 hours had been quite normal at the end of 48 hours. This observation led to the inoculation of a 48-hour mash with the chain-forming isolate. A strong qualitative test for acrolein was obtained in the distillate after the mash had incubated an additional 24 hours at 30 C. The inoculation of partially fermented mashes with the chain-forming isolate made it possible to produce acrolein in controlled laboratory fermentations.

Attempts to identify the organism have led to the concept that it is an unidentified species. A complete

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FIG. 1. Growth of acrolein bacterium as produced on tomato juice broth.

description is not within the scope of this paper. Its typical growth habit is that of long, interlaced chains, as shown in figure 1.

Table 1 summarizes some of the common physiological and morphological characteristics of the acrolein-producing organism. Similar data from the literature on other organisms which have been reported to produce acrolein are included for comparison.

Appropriate experiments served to demonstrate that the bacterium is capable of enduring the temperature and time of conversion of grain mashes, namely, 62 C for 20 minutes.

It was established that acrolein could be produced in the laboratory on either rye, bourbon, or spirits mashes. The plant mashes which were used in this work were of the latter type and contained approximately 53 per cent corn meal, 39 per cent milo meal and 8 per cent ground malt.

The experiment in which acrolein was produced by inoculating a 48-hour mash with the isolate suggested that the presence of sugars in the fresh mashes might in some way inhibit the production of the aldehyde. This premise was confirmed by adding varying amounts of glucose to 100-ml portions of a 48-hour plant mash, sterilizing the mixture and inoculating with 1 ml of a 24-hour tomato-juice-broth culture of the isolate. The results as shown in table 2 indicate that a concentration of sugar higher than approximately 0.6 per cent, expressed as glucose, completely inhibits acrolein production on this substrate. The effects of glucose will be considered later in more detail.

The effect of pH on the formation of the aldehyde was shown by adjusting portions of a 48-hour plant beer to varying pH values, sterilizing, and inoculating with the acrolein producer. The results given in table 3 show that acrolein was not produced below a pH of about 4.0. Good growth of the organism occurred as low as pH 3.5 in a tomato juice broth.

It was postulated from the results which were obtained up to this time that, in order for the bacterium to produce acrolein, it was necessary for it to attain its maximum development at a relatively late stage of the yeast fermentation. Two possible mechanisms by which this could occur were investigated: 1) contamination from wooden fermentors, and 2) the introduction of very small number of bacteria into the mash near the beginning of the yeast fermentation.

Cypress blocks measuring 1 x 1 x 4 inches were placed in flasks containing tomato juice broth. After sterilization, the broth was inoculated with the acrolein-producing bacterium and incubated. After 24 hours the blocks were removed, washed with water, steamed gently and transferred to flasks of freshly converted and yeasted mash. After 72 hours the mashes containing the blocks contained 8 ppm of acrolein. Control flasks contained none. It was concluded from these data that wooden fermentors are a potential source of inoculum of the acrolein-producing organism.

It was demonstrated that the introduction of a very small inoculum into a mash would result in the slow development of the acrolein producer and thus, in effect, produce relatively large numbers of the organism at about 24–48 hours after yeasting. One liter of a freshly-yeasted mash was inoculated with 5 ml of a 24-hour tomato-juice-broth culture of the acrolein producer. Similarly, other mashes were inoculated with 10-ml portions of 10^{-5} to 10^{-7} dilutions of the same culture of the organism. Bacterial counts and acrolein determinations were made daily. The data are presented in table 4.

It may be seen from these data that the mashes given very small inocula produced acrolein, while the mash given a heavy inoculum produced none. Maximum bacterial counts were reached in the heavily inoculated mash in 24 hours, but 72 hours were required for maximum counts with the small inocula. Only the latter mashes contained acrolein. These data make it quite clear why ordinary sanitation methods in a distillery often fail to eliminate "pepper". In order to do this, it would be necessary to reduce the bacteria in the mash to less than ½ cell per ml. Such a degree of sanitation can seldom, if ever, be realized in a distillery.

A number of antiseptics were added to inoculated

TABLE 1. *Comparison of the morphology and physiology of the acrolein bacterium with other acrolein-producing organisms*

CHARACTERISTIC	ACROLEIN BACTERIUM	*Clostridium perfringens (welchii)**	*Escherichia freundii (Citrobacter freundii)**	*Bacillus polymyxa (Bacillus amaracrylus)**
Motility	Nonmotile	Nonmotile	Motile or Nonmotile	Motile
Gram stain	Positive	Positive	Negative	Positive
Morphology	Rods occurring singly, pairs, chains	Rods occurring singly, pairs, chains	Rods occurring singly, pairs, chains	Rods occurring singly and chains
Nitrate reduction	Positive	Positive	Positive	Negative
Surface growth	Colonies small, circular, raised	Colonies circular, raised, moist	Colonies smooth, gray, shining	Colonies—lobated, circular gray-white
Spore formation	None	Spores oval central to excentric	None	Spores—ovoid central
Catalase production	Negative	—	Positive	—
Growth on litmus milk	No action	Acid coagulated	Acid may coagulate	Gas coagulated
Growth on broth	Turbid	Turbid	—	Turbid, grayish pellicle
Gelatin liquefaction	None	Liquefied	None	None
H_2S production	None	—	Positive	None
Fermentation of:				
dextrose	Acid	Acid and gas	Acid and gas	Acid and gas
mannose	Acid	Acid and gas	Acid and gas	—
arabinose	Acid	—	Acid and gas	Acid and gas
xylose	Acid	Acid and gas	Acid and gas	Acid and gas
raffinose	None	Acid and gas	Acid and gas	Acid and gas
lactose	None	Acid and gas	Acid and gas	Acid and gas
galactose	Acid	Acid and gas	Acid and gas	Acid and gas
maltose	Acid	Acid and gas	Acid and gas	Acid and gas
levulose	Acid	Acid and gas	Acid and gas	—
dextrin	None	—	—	Acid and gas
rhamnose	None	—	Acid and gas	None
inositol	None	Acid and gas	Variable	—
mannitol	None	—	Acid and gas	Acid and gas
Temperature characteristics	Optimum, 35 C; viable after 20 min at 75 C	Optimum, 35 C	Optimum, 30–37 C	Optimum, 30 C
Oxygen requirements	Microaerophilic	Anaerobic	Aerobic, facultative	Aerobic, facultative

* Data as given in *Bergey's Manual of Determinative Bacteriology*, 6th Ed., 1948, The Williams & Wilkins Co., Baltimore, Md.

TABLE 2. *Inhibition of acrolein production by sugars*

NO.	ADDED GLUCOSE	TOTAL SUGAR AS GLUCOSE	ACROLEIN
	gm/100 ml	*gm/100 ml*	*ppm*
1	0.0	0.223	420
2	0.05	0.273	436
3	0.1	0.323	394
4	0.2	0.423	280
5	0.3	0.523	128
6	0.4	0.623	0

TABLE 3. *Effect of mash pH on acrolein production*

NO.	INITIAL MASH	ACROLEIN
	pH	*ppm*
1	3.5	0
2	4.0	47
3	4.6*	212
4	5.0	162
5	5.5	108
6	6.0	100
7	6.5	100
8	7.0	110
9	7.5	0

* Unadjusted control.

mashes for the purpose of inhibiting the acrolein-producing organisms. Of these, only the quaternary ammonium compounds proved to be of any value. To 100-ml portions of a freshly-set plant mash were added the indicated amounts of the quaternary ammonium compound and of the organism. A concentration of 1–10,000 of this particular compound was quite effective in inhibiting the acrolein production. Alcohol yields were not affected to any appreciable degree. The data are given in table 5.

Agitation under aerobic or anaerobic conditions completely inhibited the production of the aldehyde. Aerobiosis, as obtained in a quiescent incubation in a shallow layer, reduced the yield of acrolein. The data are presented in table 6. Microscopic examinations of the mashes indicated that good growth occurred under all the experimental conditions. The reasons for this phenomenon have not been clarified.

Some preliminary studies have been made on sub-

TABLE 4. *Effect of amount of initial contamination on acrolein production*

ADDED BACTERIA	TIME OF ANALYSIS					
	24 Hr		48 Hr		72 Hr	
	Acrolein	Bacteria	Acrolein	Bacteria	Acrolein	Bacteria
no./ml mash	*ppm*	*no./ml mash*	*ppm*	*no./ml mash*	*ppm*	*no./ml mash*
245×10^4	0	17×10^7	0	14×10^7	0	22×10^7
49	0	23×10^3	9	23×10^5	25	13×10^7
4.9	0	7900	0	79×10^4	33	70×10^6
0.49	0	—	0	49×10^4	89	70×10^6

TABLE 5. *Inhibition of acrolein production by a quaternary ammonium compound*

CONCENTRATION OF QUATERNARY	CONTROL MASH*			INOCULATED MASH†		
	Alcohol	Final pH	Acrolein	Alcohol	Final pH	Acrolein
	per cent		*ppm*	*per cent*		*ppm*
None	7.51	4.1	0	7.44	3.9	56
1–5,000	7.42	4.4	0	7.47	4.4	0
1–10,000	7.47	4.3	0	7.29	4.2	0

* Analyzed after 72 hours' fermentation.

† Inoculated with 1 ml 10^{-6} dilution of a 24-hour broth culture of acrolein producer.

TABLE 6. *Influence of oxygen tension and agitation on acrolein production*

NO.	TREATMENT*	ACROLEIN
		ppm
1	Incubated anaerobically under mineral oil. 250-ml flask sealed with parafilm. Not agitated	232
2	Incubated aerobically in 1-L flask	22
3	Incubated aerobically. Agitated in 1-L flask	0
4	Incubated anaerobically under N_2. Agitated in 1-L flask	0
5	Incubated anaerobically under CO_2. Agitated in 1-L flask	0

* Portions, 100 ml of sterile 48-hour plant mash.

strates other than grain mash for the formation of acrolein. Attempts to produce the aldehyde on Voisenet's substrate or on tomato juice broth containing added glycerol were completely negative. The work of Reynolds *et al.* on *Escherichia* (*Citrobacter*) *freundii* could be duplicated readily with his organism, but not with ours. Since it was known that acrolein could be produced on a 48-hour plant beer, a sample of the latter was filtered, and the filtrate inoculated with the acrolein producer. No acrolein was produced on this clear filtrate, although the aldehyde was produced on the unfiltered mash. The presence of the grain residue, therefore, is necessary for the production of the compound. That the grain residue was not functioning in a purely mechanical manner was shown by substituting washed sawdust, Hyflo or Vermiculite for the residue. No acrolein was produced on substrates containing these materials. Mash residue was used as a constituent of the substrate in many subsequent experiments.

TABLE 7. *Effect of glucose on acrolein production in a "synthetic" substrate**

NO.	GLUCOSE	ACROLEIN
	per cent	*ppm*
1	0	36
2	0.1	29
3	0.5	376
4	1.0	500
5	2.0	10

* Substrate contained: washed grain residue from 100 ml 48-hour mash; 40 per cent tomato juice (sugar free); 1 per cent yeast extract; 3 per cent glycerol; 0.5 per cent salts; H_2O to 100 ml.

It was shown by means of appropriate experiments that tomato juice broth containing glycerol and glucose could be substituted for the mash filtrate. In table 7 are shown the effects of varying amounts of glucose in a substrate containing washed grain residue, tomato juice, yeast extract and 3 per cent glycerol.

The acrolein production passes through a maximum at a glucose concentration of about 1 per cent. Higher or lower concentrations of sugar resulted in lower yields of the aldehyde. The growth of the organism was considerably reduced in those substrates containing little or no glucose, and this may account for the reduced amounts of acrolein. Possibly the organism can utilize glycerol much more efficiently in the presence of some glucose; but if sufficient glucose is present to supply the needs of the organism, then no glycerol is metabolized. Table 8 shows the effects of varying amounts of glycerol in the same grain residue-tomato juice substrate containing 1 per cent glucose. Increasing amounts of glycerol resulted in higher yields of acrolein.

Since the grain residue contains yeast cells and insoluble particles derived from the grains, it seemed feasible to use yeast cells in place of the grain residue.

TABLE 8. *Effect of glycerol on acrolein production in a "synthetic" substrate**

NO.	GLYCEROL	ACROLEIN
	per cent	*ppm*
1	0	0
2	0.1	0
3	0.5	7
4	1.0	388
5	2.0	402
6	3.0	480

* Substrates contained: washed residue from 100 ml 48-hour mash; 40 per cent tomato juice (sugar free); 1 per cent yeast extract; 1 per cent glucose; inorganic salts; H_2O to 100 ml.

A substrate having the composition: 20 per cent moist yeast cells, 40 per cent filtered tomato juice, 3 per cent glycerol, 1 per cent yeast extract and 1 per cent glucose was prepared and inoculated with the acrolein producer. A yield of 50 ppm of acrolein was produced.

Acrolein was produced on a substrate containing the insoluble fraction of an unfermented grain mash. A bourbon grain mash was cooked and converted in a manner similar to that used in fermentation studies. After filtration and washing with hot water, the insoluble fraction was added to a substrate containing tomato juice, yeast extract, glucose, and glycerol. Twenty-four hours after inoculation with the acrolein producer, the substrate contained 55 ppm of acrolein.

Confirmatory evidence that the above effect was not mechanical was found by autoclaving the grain residue twice. A residue which was autoclaved only once, in combination with the liquid phase of the substrate, gave a yield of 150 ppm of acrolein. A residue which was autoclaved twice, once alone and once after being added to the liquid phase, gave no acrolein. No explanation has been found for the role of the solids in the formation of acrolein.

Discussion

The organism which has been isolated and studied in this investigation is distinctly different from those previously reported to produce acrolein. It also is distinct morphologically from those organisms studied by Serjak, Day, Van Lanen, and Boruff (1953). Further investigation will be required to determine whether the bacterium reported here and those reported by the above investigators are related or whether they belong to different genera.

The factors influencing the formation of "peppery" whiskey, while not unique, are such as not to be readily apparent. This may account for the lack of information concerning the phenomenon and for the many fanciful explanations which have existed in the industry. It also is quite evident that the factors influencing the formation of acrolein by this bacterium are different from those which influence the formation of the compound by other organisms. The nutritional requirements of the bacterium reported here are much more critical than those of other acrolein producers. Little or no growth of the organism occurred when it was inoculated into Voisenet's, Humphreys', or Reynolds' substrates. All of the previously reported acrolein producers apparently are capable of utilizing glycerol as the sole source of carbohydrates. The organism used in these studies requires fermentable sugars in addition to the glycerol. It is interesting to note that Warcollier and Le Moal (1932) were unable to effect the conversion of glycerol to acrolein using the cultures which they isolated from ciders containing the aldehyde.

An inspection of table 8 will reveal that a nonlinear relationship exists between the available glycerol and the acrolein which is produced. Increased amounts of glycerol operate in a manner other than simply furnishing additional raw material for acrolein production.

Summary

"Peppery" whiskey is caused by the presence of acrolein, which in turn may be produced as the result of the metabolism of an unclassified bacterium. Some of the factors which influence the production of the aldehyde are the pH of the substrate, oxygen tension, agitation, presence of yeast cells and/or mash residue, fermentable sugars, and glycerol.

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