Acrolein Production by Bacteria Found in Distillery Grain Mashes

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Acrolein not infrequently is formed during alcoholic fermentation or during the storage and maturation of alcoholic products. It was first observed by Voisenet (1910) in an investigation of wine having a bitter flavor. In his initial report and in subsequent publications (1911, 1911a, 1913, 1914) Voisenet demonstrated that acrolein was always present in wines having a bitter character and, also, that such wines contained a rod-shaped bacterial contaminant which he named *Bacillus amaracrylus*. This organism, obtainable from water, was considered originally to be related to the colon typhoid group and was found to produce bitterness in normal wines and acrolein in a peptone-glycerol-salts medium. *B. amaracrylus* was further characterized by Voisenet (1918) and is now listed in *Bergey's Manual* (Breed, Murray and Hitchens, 1948) under *Bacillus polymyxa*.

The development of bitterness in wines and brandies containing acrolein, which *per se* is not bitter, has been explained recently by Rentschler and Tanner (1951) as resulting from a combination of acrolein with polyphenol compounds such as the tannins found in grape and barrel extracts. These investigators separated and identified acrolein as the 2-4 dinitrophenylhydrazine derivative from brandies having a pungent, stinging character. High tannin wines were found to give marked bitterness upon the addition of acrolein whereas low tannin wines were not changed.

Humphreys (1924) reported the production of acrolein by *Clostridium welchii* (*perfringens*) and pointed out that the ability to produce acrolein might be useful in the identification of this species. Warcollier and associates (1932, 1934) demonstrated the production of acrolein in cider, brandy and pear juice by *Cl. perfringens*.

The production of acrolein by four strains of *Escherichia* (*Citrobacter*) *freundii* and one strain of aerobacter was noted by Reynolds *et al.* (1939). Dimedon (dimethyldihydroresorcinol) was employed as a fixative in the determination of acrolein. Mickelson (1939), in a further study of this group of organisms, explored the possibility of acrolein being an intermediate in the formation of trimethylene glycol from glycerol but concluded that it was more likely an end product of glycerol fermentation. It is apparent from the above literature that at least four species of bacteria are capable of producing acrolein. In most instances acrolein was believed to be a unique fermentation product and of definite significance in characterizing the particular species involved.

When acrolein appears in grain alcohol fermentations it is called "pepper". The term "pepper" probably originates from the pronounced irritant and lachrymatory properties of acrolein, especially when it is distilled from affected beers. "Pepper" in commercial distillery operations occurs sporadically; it may appear in an occasional fermentor or in a series of fermentors set over an extended period of time (See Nettleton, 1913; Mills *et al.* 1953). During a "pepper" period, alcohol distillates range in acrolein content from a trace to as much as 100 ppm or more. Since as little as 10 ppm in a whiskey (low-proof) distillate is readily detectable organoleptically, corrective measures are necessary. Usually, the product containing acrolein is redistilled to high-proof spirits during which acrolein concentrates in and is separated with the "aldehyde" fraction. Thus, no quality reduction is incurred by "pepper" if the whiskey is redistilled to spirits, but the redistillation and necessity of converting more expensive whiskey grain bills to high proof spirits entail additional operating costs.

In view of previous demonstrations that acrolein was formed in other alcoholic fermentations by contaminating bacteria, numerous attempts were made in our laboratories to demonstrate the presence of these known acrolein-producing species during "pepper" periods and also to produce acrolein with these species in laboratory-scale grain alcohol fermentations. All such attempts were unsuccessful. Moreover, no gross differences could be found in the numbers or morphology of the bacterial flora of grain mashes during normal distillery operations and "pepper" periods. In both instances the organisms, as is generally assumed, appeared to be lactic types and none produced acrolein when inoculated into grain mashes. Some isolates from both sources, however, were found capable of producing acrolein in a medium composed of grain stillage and glycerol.

The present study, which is divided into two parts, was undertaken to better characterize the bacterial flora of grain mashes during a "pepper" period and to explore some of the factors which predispose to acrolein formation. Part one is concerned with the identification of 265 organisms isolated largely from

acrolein-containing beers and part two describes experiments on the production of acrolein in laboratory-scale grain alcohol fermentations.

Experimental Methods

End fermentor samples after proper dilution in sterile water blanks were inoculated into tomato juice agar (TJA) shake cultures as described by Garey *et al.* (1945). The composition of TJA medium was as follows:

Filtered juice of canned tomatoes	400 ml
Difco yeast extract	10 g
Glucose	10 g
Salt solution A	5 ml
Salt solution B	5 ml
Distilled water	600 ml
Agar	15 g
Adjusted to pH 6.9	

Salt solution A contained 25 g each of mono- and dibasic potassium phosphate per 250 ml of distilled water. Salt solution B contained 10 g of magnesium sulfate heptahydrate, and 0.5 g each of sodium chloride, ferrous sulfate heptahydrate, and manganous sulfate tetrahydrate per 250 ml of distilled water.

After 24 hours' incubation at 37 C, the agar from tubes containing well-isolated colonies was "blown" into sterile Petri dishes and representative colonies were transferred by needle to tomato juice broth (TJB). All isolates were replated in TJA medium and, thereafter, were carried in TJA stabs containing 5 g glucose per liter. It was found necessary to transfer at intervals of about 2 weeks to maintain the viability of isolates.

Growth at various temperatures was determined in TJA medium. Readings were made after 10 days at 10 C and 22 C, after 72 hours at 37 C and after 6 days at 45 and 50 C. Acid production, as indicated by the final pH attained, was determined in TJA medium containing 3 per cent glucose which was added aseptically after sterilization. Readings were made after 10 days' incubation at 37 C.

Fermentation of sugars and related carbon compounds was studied in tryptone-yeast extract medium (TY) containing 10 g tryptone, 10 g yeast extract, 10 g of the respective carbon source and 5 ml of salt B per liter. In addition, TJB medium was used with glucose. Xylose, arabinose, maltose, and melibiose were sterilized by Seitz filtration and added aseptically to basal TY medium. Durham tubes were inserted to detect gas production and readings were made after 10 days' incubation at 37 C. Litmus milk was prepared from Difco Bacto skim milk. Reduction and acid production were noted after 6 days' incubation at 37 C.

The ability of isolates to produce acrolein was determined in centrifuged grain stillage to which 20 g of glycerol was added per liter. This medium was adjusted to pH 5.5, tubed in 30-ml quantities, and autoclaved at 15 pounds for 20 minutes. After 48 hours' incubation at 37 C, a 15-ml aliquot of the fermented medium was distilled and the first 4 ml of distillate was taken for the acrolein determination. Acrolein was determined by the method of Circle *et al.* (1945). Glycerol was determined by the method of Amerine and Dietrich (1943).

Laboratory-scale grain mashes were prepared by weighing 91.3 g of ground grain and 0.8 g ground malt into a 1-liter Florence flask. Four hundred and twenty-five ml of water at 140 F was added to each and the temperature was raised with agitation to 180 F. The mashes were then cooked at 20 pounds for 45 minutes, cooled to 146–148 F. and 8 g of ground malt was added to each flask. After 45 minutes of conversion, during which the mashes were agitated intermittently, the flasks were cooled to 75 F. and the pH adjusted to 4.8 with lactic acid. The final weight of each mash was brought to 571 g with water and 15 ml of a 24-hour yeast culture was added. Acrolein-producing organisms were introduced as indicated and all mashes were fermented 72 hours at 90 F.

Experimental Results

Table 1 shows the locations routinely sampled in our Peoria distillery and typical bacterial (TJA) counts at these locations. It may be seen that barley malt meal, in both dry and slurry form, carries a high bacterial load. Converted mash, which is comprised of grain meal cooked under pressure and thereafter cooled and saccharified with malt slurry at 148 F for 30 minutes, is substantially reduced in bacterial numbers. There is a slight increase in the number of bacteria in mash as it is pumped through the vacuum cooler to the fermentors. Mash is diluted in the fermentors by the addition of about 25 per cent of sterile stillage which accounts for the slight lowering of the bacterial count at this stage. Bacterial numbers remain low during the major portion of the fermentation. However, in the late stages of the fermentation, when the sugar fermentable by yeast is almost com-

Table 1. *Bacterial counts at various steps of grain mashing and fermentation*

SAMPLE	BACTERIAL COUNT
	*millions/g**
Corn meal	0.7
Barley malt meal	6.5
Barley malt slurry	1.7
Converted corn-malt mash	0.0001
Converted mash from vacuum mash cooler	0.0013
Set fermentor, corn-malt mash	0.0006
24-hr fermentor, corn-malt mash	0.17
72-hr fermentor, final beer	200

* Tomato juice agar medium.

TABLE 2. *Formation of glycerol in a commercial grain alcohol fermentation*

HOURS OF FERMENTATION	GLYCEROL
	mg/100 ml mash
0 (Set fermentor)	195*
8	181
16	364
24	447
30	458
48	500
55 (Final fermentor sample)	562

* Includes the glycerol introduced with the seed yeast.

pletely utilized, the bacterial count increases rapidly, as indicated by the 72-hour count of 200 million per ml. As stated previously, bacterial counts during "pepper" periods are not significantly different from those found during normal operations, although it is fairly common to find lower than normal counts on these occasions.

The formation of glycerol during alcoholic fermentation is well known. Representative data obtained from the progressive analysis of a commercial-scale grain alcohol fermentation are shown in table 2. While acrolein production in a given fermentation might be expected to cause a reduction in glycerol content, analyses of a number of acrolein and non-acrolein fermentors failed to establish a quantitative relationship between glycerol utilization and acrolein production. Apparently, only a small percentage of the glycerol contained in a fermenting grain mash is metabolized by the bacteria present.

While a trace of acrolein occasionally may be formed during the active stage of yeast fermentation (18–30 hours), most commonly, if formed, it begins to appear during the period closely corresponding with the sharp increase in bacterial numbers. For this reason the present isolations were all made from final fermentor samples. Data on the fermentors sampled and the numbers of isolates obtained from each are shown in table 3.

When replated on TJA medium to check their purity, most of the isolates appeared in the form of one or more of four different smooth, subsurface colony types. The predominant type was ellipsoidal or lens-shaped, the others spindle, cloverleaf or collar-button types, depending upon the direction of outgrowths from the original lens-shaped colony. One round Rough type also was frequently observed. Colonies ranged from about 0.25 to 2.0 mm in size.

All isolates were gram positive (gram variable in older cultures) nonmotile, nonsporulating, catalase-negative, microaerophilic rods which occurred singly, in pairs, and in short chains. No streptococci or obligate anaerobes were found, although various media and culture conditions capable of supporting the growth of such organisms were used. Thus, the entire flora appeared to be limited to members of the genus *Lactobacillus*.

TABLE 3. *The source of bacterial isolates identified*

FERMENTOR NO.	MASH BILL	ACROLEIN IN DISTILLATE	NO. STRAINS ISOLATED
		ppm	
3	Milo-malt	4	27
4	Milo-malt	6	15
5	Milo-malt	2	30
12	Milo-malt	3	32
14	Milo-malt	12	25
15	Milo-malt	5	17
17	Corn-malt	0	14
16	Corn-malt	4	9
18	Corn-malt	1	33
19	Corn-malt	1	16
17-1	Corn-malt	2	57
Total			265

Based upon the morphological and physiological characteristics summarized in tables 4 and 5, 248 of the isolates were arranged into one homofermentative group (A) and seven heterofermentative groups (B–H). The remaining 17 isolates could not be classified. The frequency of appearance of groups A–H in the fermentors sampled was as follows:

	Lactobacillus Group							
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*
No. of fermentors positive of 11 sampled	7	7	10	4	8	2	8	10

The characteristics of group A organisms are in good general agreement with those ascribed (*Bergey's Manual*) to *Lactobacillus plantarum*. The only differences noted were that group A organisms were somewhat shorter, failed to coagulate litmus milk although they produced some acid, and varied in their fermentation of xylose. Neither *L. plantarum* nor any of the group A isolates produced acrolein from stillage-glycerol medium.

The remaining groups (B through H) were heterofermentative, particularly on glucose substrates. However, gas production varied markedly with the basal medium, being very low or negligible in TY medium and abundant in TJB medium, or the medium of Krehl *et al.* (1943). Since each of these media supported good to excellent growth, the existence in the latter two media of a factor necessary for the breakdown of triose compounds is suggested. Supplementing the TY medium with thiamine and vitamin B_6 compounds failed to increase the amount of gas produced.

Group B organisms resemble most closely *Lactobacillus brevis*. However, this group differed from *L. brevis* in that few strains produced acid in litmus milk. As with some strains of *L. brevis*, sucrose, lactose and raffinose were not fermented while mannose was fer-

TABLE 4. *Characteristics of Lactobacilli isolated from grain mashes*

GROUP	NO. OF ISOLATES	AVERAGE CELL SIZE	GROWTH IN TOMATO JUICE BROTH C					FINAL pH REACHED IN TOMATO JUICE BROTH		REACTION IN LITMUS MILK		ACROLEIN FORMATION IN STILLAGE GLYCEROL MEDIUM		SPECIES RELATIONSHIP
			12	22	37	45	50	Average	Range	Reduction	Acid	Negative	Positive	
		microns												
A	20	0.6 × 1.8	W+	+	+	±	—	3.35	3.32–3.39	+	+	20	0	*L. plantarum*
B	50	0.5 × 1.7	W+	+	—	—	—	3.80	3.57–3.83	Partial*		50	0	*L. brevis*
C	39	0.5 × 1.6	—	+	+	±	—	3.53	3.45–3.63	Partial	W+	36	3	*L. buchneri*
D	9	0.45 × 1.8	—	+	+	±	—	3.50	3.43–3.55	Partial	—	8	1	*L. buchneri*
E	30	0.6 × 1.8	—	+	+	±	—	3.54	3.47–3.62	Partial	W+	28	2	*L. buchneri*
F	7	0.75 × 2.2	—	+	+	+	—	3.45	3.43–3.57	Partial	W+	3	4	*L. pastorianus*
G	36	0.5 × 1.8	—	+	+	±	—	3.52	3.45–3.65	Partial	—	36	0	New species
H	57	0.7 × 2.2	—	W+	+	±	—	3.48	3.43–3.63	Partial	+	2	55	New species

W+ = Weakly positive growth or acid production.
* A few isolates produced acid.

TABLE 5. *Fermentation of various carbon sources by Lactobacilli from grain mashes*

GROUP	NO. OF ISOLATES	CARBON SOURCES																			SPECIES RELATIONSHIP
		Glucose		Mannose		Galactose	Fructose	Arabinose	Xylose	Sucrose	Lactose	Maltose	Trehalose	Melibiose	Raffinose	Rhamnose	Inulin	Salicin	Mannitol	Glycerol	
		A	G	A	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	
A	20	+	—	+	—	+	+	+	±	+	+	+	+	+	+	—	W+	+	+	W+	*L. plantarum*
B	50	+	+	±	—	+	+	+	+	—	—	+	—	+	—	—	—	—	—	W+	*L. brevis*
C	39	+	+	+	—	+	+	+	+	+	+	+	—	+	+	—	—	—	*	W+	*L. buchneri*
D	9	+	+	±	—	+	+	+	+	+	—	+	—	+	+	—	—	—	—	—	*L. buchneri*
E	30	+	+	+	†	+	+	+	+	+	+	+	+	+	+	—	*	*	*	*	*L. buchneri*
F	7	+	+	+	†	+	+	+	—	+	W+	+	±	+	+	—	—	—	W+	W+	*L. pastorianus*
G	36	+	+	W+	†	+	+	+	—	+	—	+	+	+	+	—	—	—	W+	W+	New species
H	57	+	+	±	—	+	—	+	—	+	+	+	—	+	+	—	—	—	—	*	New species

A = acid; G = gas. ± = Variable acid production. W+ = Weak acid production.
* A few isolates produced acid from these carbon sources.
† A few strains of each of these groups were weak gas producers.

mented only by some of the members of the group but without gas production.

Groups C, D, and E are closely related to *Lactobacillus buchneri* and similar to one another with respect to temperature relationships and the final pH produced by each in TJB medium. All the isolates of each group produced acid from arabinose, xylose, fructose, galactose, maltose, and raffinose and all failed to ferment rhamnose. Groups C and D produced acid from lactose and a weak acid reaction in litmus milk, whereas group E isolates did not produce acid in these media. Inulin, salicin, mannitol, and glycerol were fermented by a few group E isolates but by none of the group D isolates. A weak glycerol fermentation was observed with some group C isolates and few fermented mannitol. Eight of the 72 group C, D, and E organisms produced acrolein in stillage-glycerol medium.

Group F organisms are closely related to, if not identical with, *Lactobacillus pastorianus*. These isolates did not occur in the long chains generally characteristic of *L. pastorianus* but their average length was greater than that found with the other groups except group H which were about the same size. Group F organisms and *L. pastorianus* produced acid and gas from glucose and acid from arabinose, fructose, galactose, maltose, sucrose and raffinose. Trehalose, fermented by *L. pastorianus*, was attacked by some but not all group F organisms. Xylose, inulin, and rhamnose which are not fermented by *L. pastorianus* were not utilized by this group. However, the group partially reduced litmus milk and developed a slight acid reaction, while *L. pastorianus* produced acid in litmus milk. Four of the seven group F organisms produced acrolein in stillage-glycerol medium.

Groups G and H differ appreciably from previously described species of lactobacilli. Group G organisms, while similar in several respects to *L. pastorianus*, were slightly smaller in average size under comparable growth conditions and displayed a lower growth temperature. Further, none of the group G isolates, in contrast to *L. pastorianus*, formed acid in either lactose

medium or litmus milk, but all actively fermented trehalose. None of the 36 isolates produced acrolein in stillage-glycerol medium.

Group H organisms were separable from the previous groups by their inability to ferment xylose, fructose, and trehalose, in addition to negative reactions on rhamnose, inulin, salicin, and mannitol. Only a few members of this group produced acid from mannose and glycerol, and both groups G and H bear some relationship in general morphology, low maximum growth temperatures and weak gas production to the lactobacilli found by Niven *et al.* (1949) to cause a green discoloration of sausage. However, each of these types are distinguishable from the others by their reactions on various sugars. By the same criteria, groups G and H are distinct from *L. malefermentans* N.sp. (Russel and Walker, 1953) and *L. parvus* N.sp. (Russel and Walker, 1953a).

Although 55 of 57 group H organisms produced acrolein in stillage-glycerol medium, only a small percentage fermented glycerol with the production of acid. The low fermentative activity of this group on glycerol suggested that acrolein might not arise from the fermentation of glycerol. To investigate this possibility, the action of representative group H isolates on glycerol and glucose, separately and in combination, was determined, using conventional Warburg techniques. Preliminary results showed that glucose was readily oxidized without acrolein formation, whereas glycerol (initial concentration, 15 mg per ml) was not oxidized but approximately 1 per cent was converted to acrolein during a 1-hour incubation period at 30 C. With glucose and glycerol in combination, no acrolein was formed and the rate of oxygen uptake was unchanged over that with glucose alone. Thus, in the presence of glucose, glycerol is not converted to acrolein by this group of lactobacilli.

TABLE 6. *Acrolein organisms in distillery raw materials*

MATERIAL TESTED	NO. OF SAMPLES TESTED	SAMPLES CONTAINING ACROLEIN-PRODUCING ORGANISMS*
		per cent
Well water	15	0
Yeast inoculum	32	0
Backstillage	22	0
Lactic culture (*L. delbruckii*)	4	0
Barley malt:		
Malt company A	184	63
Malt company B	59	57
Malt company C	32	62
Malt company D	44	27
Milo (grain sorghum) dust	30	60
Milo (grain sorghum) meal	29	10
Corn meal	26	20
Barley malt saccharified grain mashes:		
Rye	9	25
Corn	24	58
Milo	32	85

* Tests conducted in stillage-glycerol medium.

TABLE 7. *Effect of inoculum size upon the production of acrolein by* Lactobacillus *strain no. 208 in milo (grain sorghum) mashes*

INOCULUM ADDED	CELLS OF STRAIN NO. 208/ml OF MASH	FINAL pH	ACROLEIN IN DISTILLATE
per cent by volume			*ppm*
None		4.19	None
0.0001	0.2×10^3	4.25	20
0.001	2.0×10^3	4.25	10
0.01	2.0×10^4	4.17	Trace
0.1	2.0×10^5	4.10	None
1.0	2.0×10^6	4.09	None

Subsequent studies were directed toward the manner in which acrolein-producing organisms are brought into the distillery and, after introduction into the distillery, the factors which lead to acrolein formation. Table 6 shows the incidence of acrolein-producing organisms in distillery raw materials. It will be noted that grain and grain dust are frequently contaminated with these types with over half the barley malt containing acrolein-producing bacteria by the stillage-glycerol test. Since malt is the only raw material not sterilized in the mashing process, it is the most probable external source of acrolein-producing organisms.

Using a typical group H isolate (no. 208), the influence of inoculum size upon acrolein production in grain mashes undergoing alcoholic fermentation was investigated. As stated previously, all prior attempts to produce acrolein in laboratory-scale grain mashes had been unsuccessful. Table 7 shows the results of a typical experiment. It will be noted that, contrary to expectations, only the low inoculum levels led to acrolein formation. By controlling the inoculum size in subsequent experiments it was found that acrolein could be produced in laboratory-scale mashes with only an occasional failure. This observation made possible the study of numerous variables on acrolein formation in alcoholic fermentations.

Table 8 shows the production of acrolein in mashes fermented with different distillers' yeast cultures. All mashes were prepared in the same manner and inoculated with 0.001 per cent by volume of a 24-hour culture of *Lactobacillus* strain no. 208. Alcohol yields varied from 6.26 proof gallons per 56 lbs of grain (dry weight basis) with yeast no. 1 to 6.09 proof gallons with yeast no. 6. Acrolein in the distillates ranged from 13 ppm with the highest yielding yeast to 1 ppm with the lowest. Thus, more efficient yeasts which better utilize fermentable sugars led to greater acrolein production. This experiment confirms plant-scale experience, since acrolein generally accompanies periods of high alcohol yields when fermentation efficiencies are at their peak.

TABLE 8. *Acrolein production by* Lactobacillus *strain no. 208 in milo (grain sorghum) mashes fermented with various yeast strains**

Saccharomyces cerevisiae DISTILLERS STRAINS	FINAL pH	ALCOHOL YIELD, PROOF GAL./56 LBS. OF GRAIN (DRY BASIS)	ACROLEIN IN DISTILLATE
			ppm
No. 1.....	4.03†	6.26	13
No. 2.....	3.97	6.25	10
No. 3.....	3.97	6.25	5
No. 4.....	4.00	6.13	5
No. 5.....	3.98	6.09	3
No. 6.....	3.95	6.09	1

* All mashes received 0.001 per cent by volume inoculum of 24-hr culture of *Lactobacillus* strain no. 208.

† Seventy-two-hour fermentation at 90 F.

Since both acrolein- and nonacrolein-producing organisms occur in grain mashes it became of interest to determine what effect, if any, the nonacrolein types might have upon acrolein production. Table 8 shows the results of a laboratory experiment in which corn-malt mashes were seeded with the usual 3 per cent by volume of yeast. Another set received, in addition, 0.001 per cent by volume of *Lactobacillus* strain no. 208 and the third received an equal amount of this culture and, in addition, 0.05 per cent by volume of mixed nonacrolein flora obtained from a final fermented beer. It is apparent that the mixed flora inhibited completely the production of acrolein.

DISCUSSION

The data obtained in the microbiological survey of our plant show that, 1) the numbers of bacteria in distillery raw materials are sharply reduced during the mashing process, and 2) the surviving bacteria which later develop during yeast fermentation are restricted to a homogenous group, namely, the lactobacilli. The presence of other types of bacteria such as the aerobic and anaerobic spore formers, the destruction of which would not be expected by the time and elevated temperature of saccharification (148 F for 30 minutes), were not demonstrable owing probably to the low pH of set mashes (4.8–4.6) and in the case of aerobes, the low redox potential.

Approximately 50 per cent of all the malt samples examined contained acrolein-producing organisms with malts from the four individual suppliers ranging from 27 to 63 per cent positive. Since malt is not sterilized in the mashing process and cannot be subjected to higher temperatures without loss of enzyme activity, it is probably the principal external source of acrolein-forming organisms in grain distilleries. Of course, after being introduced into distillery mashes and equipment, these types could be maintained and recycled by the incomplete sterilization of fermentors, mash lines, and so forth.

TABLE 9. *Influence on acrolein production of inoculating grain mashes with acrolein and nonacrolein-producing lactobacilli*

ACROLEIN-PRODUCING *Lactobacillus* STRAIN NO. 208, INOCULUM BY VOLUME	NONACROLEIN-PRODUCING TYPES* INOCULUM BY VOLUME	ALCOHOL YIELD, PROOF GAL/56 LBS. GRAIN (DRY BASIS)	BACTERIA COUNT AFTER 72 HR	ACROLEIN IN DISTILLATE
per cent	*per cent*		*millions/ml*	*ppm*
None	None	6.15	84	None
0.001	None	6.03	130	20
0.001	0.05	5.96	70	None

* Mixed flora from nonacrolein distillers final beer.

Considering the high frequency of occurrence of acrolein-producing bacteria in malt as well as in grains and grain dust, the question arises as to why "pepper" is not more frequently encountered in the fermentation of grain mashes. A logical explanation for the low incidence of "pepper" can be drawn from an examination of the data presented in tables 7 through 9. The relatively high saccharification temperature and the subsequent physical and chemical environment of fermenting grain mashes restrict the bacterial flora to *Lactobacillus* types such as those described above. Under normal operating conditions in a given distillery, this flora is quite reproducible, varying only in individual rates of growth and, consequently, in the numbers of each type at any particular sampling time. Likewise, the maximum bacterial population is generally attained before the fermentable carbohydrate supply is exhausted. Under this so-called "normal" growth pattern, nonacrolein types (for example groups A to G) predominate over acrolein-producing organisms as shown in table 9 and no acrolein is formed.

If, conversely, the surviving lactobacilli develop at a subnormal rate and both growth and carbohydrate metabolism are actively progressing after the fermentable carbohydrate supply is depleted, then glycerol is utilized as a carbon source with the formation of acrolein. This situation might result from lower numbers of total bacteria initially (table 7), an abnormally high ratio of acrolein to nonacrolein types, or an environment less favorable to rapid bacterial growth. These conditions are in harmony with the observation that "pepper" is more likely to occur where plant sanitation measures are only partially effective with the result that bacteria are reduced in number but not eliminated and reach maximum numbers at a later stage in the alcoholic fermentation. High-yielding yeast strains which effect an early and substantially complete removal of maltose (table 8) likewise favor acrolein production and play a role in determining whether or not this substance will be produced.

Since present grain distillery practice using malt as the saccharifying agent precludes bacteria-free operations, an occasional attack of "pepper" is probably

unavoidable. The observation of Mills *et al.* (1953) and the preliminary Warburg studies previously discussed, which show that glycerol is not converted to acrolein in the presence of glucose, suggest that "pepper" could be prevented or its production terminated by maintaining a low level of fermentable sugar in the fermenting mash. Insofar as the authors are aware, however, this method has not been tested during an actual "pepper" period.

As the number of bacteria shown to be capable of converting glycerol to acrolein increases, it becomes evident the required enzyme system is more widely distributed than previously believed. Further surveys using glycerol as the sole energy source might reveal even more species which can form acrolein. Therefore, this property can no longer be relied upon as an aid to the identification of bacteria, as has been frequently suggested.

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Summary

The bacterial flora associated with fermenting grain mashes in this survey were found to consist solely of members of the genus *Lactobacillus*.

Of the eight types consistently found, six were identical or closely related to known species of lactobacilli, while two types may represent new species.

Sixty-seven of 265 isolates were capable of acrolein production in stillage-glycerol medium. The most predominant acrolein-forming type was group H, in which all but 2 of the 57 isolates were acrolein-positive. Group H organisms are characterized also by their inability to ferment the more common sugars.

Barley malt appears to be the main external source of acrolein-forming organisms, although they are also present in other grains and grain dust.

Acrolein appears in grain mashes only under specific and seldom attained conditions involving primarily a low order of initial contamination, substantially complete sugar fermentation by the yeast, and the active growth of acrolein-producing types after the fermentable sugar supply is exhausted. Glycerol is then metabolized and acrolein is formed.

REFERENCES

Amerine, M. A., and Dietrich, W. D. 1943 Determination of glycerol in wines. J. Assoc. Offic. Agr. Chemists, **26,** 708–713.

Breed, R. S., Murray, E. G. D., and Hitchens, A. P. 1948 *Bergey's Manual of Determinative Bacteriology.* 6th Ed. Williams & Wilkins Co., Baltimore, Maryland.

Circle, S. J., Stone, L., and Boruff, C. S. 1945 Acrolein determination by means of tryptophane. A colorimetric micromethod. Ind. Eng. Chem. Anal. Ed., **17,** 259–262.

Garey, J. C., Rittschof, L. A., Stone, L., and Boruff, C. S. 1945 A study of cultural methods for the quantitative determination of bacterial populations of distillery mashes. J. Bacteriol., **49,** 307–310.

Humphreys, F. B. 1924 Formation of acrolein from glycerol by *B. welchii*. J. Infectious Diseases, **35,** 282–290.

Krehl, W. A., Strong, F. M., and Elveljem, C. A. 1943 Determination of nicotinic acid. Modifications in the microbiological method. Ind. Eng. Chem. Anal. Ed., **15,** 471–475.

Mickelson, M. N. 1939 Intermediary metabolism of coli-aerogenes bacteria. Ph.D. Thesis; Iowa State College, Ames, Iowa.

Mills, Doris E., Baugh, W. D., and Conner, H. A. 1953 Studies on the formation of acrolein in distillery mashes. Appl. Microbiol., **2,** 9–13.

Nettleton, J. A. 1913 *The Manufacture of Whiskey and Plain Spirit.* G. Cornwall and Sons, Aberdeen, Scotland, pp. 581–582.

Niven, C. F. Jr., Castellani, A. G., and Allanson, Virginia. 1949 A study of the lactic acid bacteria that cause surface discolorations of sausages. J. Bacteriol., **58,** 633–641.

Rentschler, H., and Tanner, H. 1951 Formation of bitterness in red wines. Acrolein in beverages and the relation to the formation of bitterness in wines. Mitt. Gebiete Lebensm. Hyg., **42,** 463–475.

Reynolds, H., Hoehn, W. M., and Werkman, C. H. 1939 Occurrence of acrolein as an intermediate during the fermentation of glycerol by the coli-aerogenes bacteria. Iowa State Coll. J. Sci., **13,** 275–277.

Russel, C., and Walker, T. K. 1953 *Lactobacillus malefermentans* n. sp. isolated from beer. J. Gen. Microbiol., **8,** 160–162.

Russel, C., and Walker, T. K. 1953a *Lactobacillus parvus* n. sp. isolated from beer. J. Gen. Microbiol., **8,** 310–313.

Voisenet, E. 1910 Formation d'acroleine dans la maladie de l'amertume des vins. Compt. rend. (Acad. Sci.), **150,** 1614–1616.

Viosenet, E. 1911 Sur un ferment de l'amertume des vins agent de dehydration de la glycerine. Compt. rend. (Acad. Sci.), **153,** 363–365.

Voisenet, E. 1911a Considerations nouvelles sur la maladie de l'amertume des vins ses rapports avec la fermentation acrylique de la glycerine. Compt. rend. (Acad. Sci.), **153,** 898–900.

Voisenet, E. 1913 Nouvelles recherehes sur un ferment des vins amers. Compt. rend. (Acad. Sci.), **156,** 1181–1182.

Voisenet, E. 1914 Sur un ferment contenu dans les eaux agent de deshydration de la glycerine. Ann. Inst. Pasteur, **28,** 807–818.

Voisenet, E. 1918 Sur une bacterie de l'eau vegetant dans les vins amers capable de deshydrater la glycerine (glycero-reaction). Ann. Inst. Pasteur, **32,** 476–510.

Warcollier, G., and Le Moal, A. 1932 The accidental presence of acrolein in the distilled liquor from cider. Compt. rend. (Acad. Sci.), **194,** 1394–1396.

Warcollier, G., Le Moal, A., and Tavernier, J. 1934 The accidental presence of acrolein in cider, brandy and pear juice. Its formation from glycerol. Comp. rend. (Acad. Sci.), **198,** 1546–1548.